Genotoxic Effects of PFOA and Fluoranthene on Blue Mussels (*Mytilus edulis)*

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Abstract

Perfluorooctanoic acid (PFOA) and fluoranthene are organic pollutants with a widespread environmental occurrence. They are both present in the environment, and are known to concentrate and bioaccumulate in organisms, PFOA in general, fluoranthene in non-vertebrates. There are several studies showing toxic effects in marine organisms exposed to PFOA or fluoranthene. However, there is a lack of studies addressing the genotoxic effects of these contaminants. In this study, DNA damage and alteration of gene expression were investigated in blue mussels (*Mytilus edulis*) exposed *in vivo* to PFOA, fluoranthene and a mixture of the two. DNA strand breaks in haemocytes were analysed using the comet assay. The sensitivity of the assay was further improved by implementing the lesion specific enzyme FPG, to detect oxidative DNA damage. Gene expression analysis of *Mt10, p53, RNA helicase and Krs*, using RT-qPCR, were performed to assess the genotoxic effects of the compounds on gill tissue.

There was substantial DNA damage in all exposure groups, including vehicle control and unexposed mussels. The amount of DNA damage in unexposed samples masked the effect of the exposure on DNA damage, and a comparison of exposed an unexposed mussels gave no clear results. No significant increase in DNA damage was observed in *M. edulis* compared to the vehicle control. Digestion with FPG caused a significant increase in oxidative DNA damage for mussels exposed to PFOA after 16 days, only. Gene expression analysis indicated oxidative stress as there was a significant up-regulation of *p53,* for mussel exposed to PFOA and mussels exposed to fluoranthene, and *Krs* for mussel exposed to PFOA. There was no significant alteration of *Mt10* or *RNA helicase.* The results from the comet assay and RT-qPCR gave no indication of increased genotoxicity from a combined exposure to PFOA and fluoranthene.

A high inter-individual variance in vehicle control (ethanol exposure only) was evident at the beginning of the experiment using the comet assay, as well as increased expression of *Mt10* and *p53*. It is therefore possible that ethanol exposure induced genotoxic effects. Since the inter-individual variance was reduced after 8 and 16 days, and the up-regulation of *p53* and *Mt10* only was transient, it is possible that mussels adapted to the ethanol exposure.

Abbreviations

ANOVA	Analysis of variance
AP sites	Apurinic/apyrimidinic sites
Bap	Benzo[a]pyrene
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
Cp	Crossing point
Ct	Threshold value
Cu^{+}	Copper
dH2O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	double stranded DNA
EDTA	Ethylenediaminetetraacetic acid
EF-1	Elongation Factor-1
FPG	Formamidopyrimidine-DNA- glycosylase
H_2O_2	Hydrogen peroxide
K_{ow}	Octanol-Water Partition Coefficient
Krs	Stress responsive kinase
LMP	Low melting point
mRNA	Messenger RNA
Mt	Metallothioneins
NaCl	Natrium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NIPH	Norwegian Institute for Public Health

NIVA	Norwegian Institute for Water Research
NTC	No template control
Oligo-dT	deoxy-thymine nucleotides
PFC	Perfluorinated compounds
PAH	Polycyclic aromatic hydrocarbons
PBS	Phosphate buffer saline
RNA	Ribonucleic acid
RT-qPCR	Real time quantitative polymerase chain reaction
RT	Room temperature
SB	Strand break
ssDNA	single strand DNA
SSBs	single strand breaks

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1. Introduction

1.2 Background

During the last decades, marine ecosystems have been subjected to increased contamination from organic compound, like polycyclic aromatic compounds (PAHs) and perfluorinated compounds (PFCs) due to anthropogenic activity. Pollution by these xenobiotic leads to environmental stress in aquatic environments, and are believed to be able to alter the physiological processes of living systems (Hylland et al. 2006). Concern for environmental and human health has lead to considerable interest in monitoring the effects of pollution in aquatic ecosystems. Of special concern are genotoxic chemicals (Wurgler and Kramers 1992). Damage to the genetic material may result in mutations, carcinogenesis, teratogenesis or tumour initiation (Mitchelmore and Chipman 1998b).

Even though there is comparatively good understanding of the toxic effect of single substances, the knowledge of the toxicity of binary or complex mixtures is more limited. Mixtures of chemicals in aquatic environments may cause toxicity due to additive or synergetic interactions between the compounds, or the adverse outcome may be reduced due to antagonistic effects (Donnelly et al. 1995). Research on the combined toxic effects of multiple chemicals is clearly more challenging than of single substances (Benedittе et al. 2007).

Many organic contaminants are genotoxicants that can modify the structure and integrity of DNA, and further damage DNA (Shugart 1995) through the generation of intermediates of greater genotoxicity formed via biotransformation during cellular processes (Shugart 1995; Newman 1998; Mitchelmore and Chipman 1998ab). Chemical compounds can interact with DNA and thereby cause DNA modifications such as DNA base modification, strand breaks, depurination and cross-linkages. As an indirect effect, species exposed to pollutants may respond with increased production of reactive oxygen species (ROS) (Winston et al. 1996), including hydroxyl radicals ($OH^{\bullet}$), hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\bullet-}$) radicals. These molecules may cause genotoxic effect through oxyradical damage to cellular material such as DNA (Ward et al. 1988). Damage to DNA may have deleterious effects at the cell (Cajaraville et al. 2003) and tissue levels (Reichert et al. 1998), which consequently may affect the health of the individual (Hylland et al. 2003).

Responses to stress stimuli, e.g. from chemicals, are supported by alteration in the gene expression, where a gene can be alternatively induced or suppressed depending on its physiological role (Farr and Dunn 1999). Transcriptional regulation is reflected by phenotypic alterations due to variation of the synthesis of mRNA and therefore changes in expression of individual proteins (Luedeking and Koehler 2004). Transcriptomic approaches have been successfully applied to unveil the molecular mechanism of adaption to both natural and chemical stressors (Venier et al. 2006; Banni et al. 2007). The transcriptional profile of specific genes can therefore be utilized to investigate the effects of exposure to organic pollutants in sentinel organisms. The application of mRNA profiling can potentially provide signatures unique to toxicant mode of action, as well as an early warning to changes in higher levels of biological organization (Dondero et al. 2006a; Venier et al. 2006).

For the validation of the genotoxicity of PFOA and fluoranthene, two different biological endpoints were used in the current study: DNA damage and modulation of gene expression. The DNA damage was evaluated using the comet assay (Östling and Johanson 1984; Gutzkow et al. 2013), while the assessment of gene expression was performed using quantitative reverse transcription PCR (RT-qPCR).

1.2 Mytilus edulis

One of the species most extensively used as a sentinel organism to detect environmental damage and/or stress are marine mussels (Goldberg 1986). The blue mussel, *Mytilus edulis,* has been widely used as an indicator species for assessing pollution by chemical substances (Goldberg et al. 1978). *M.edulis* has many advantages as an indicator species as they are ubiquitous in the Northern Atlantic, are long-lived and sedentary filter-feeders, inhabiting coastal and estuarine areas. Blue mussels feed by filtering particles from seawater, thereby continuously pumping large amount of water, which can lead to bioaccumulation of toxic substances in their body. Due to mussels being an important food source for other animals, the bioaccumulation in blue mussels could potentially lead to accumulation and biomagnification of xenobiotics in marine food chains (Widdows and Donkin 1992).

Mussels possess an open circulatory system with “blood” called haemolymph, which bathes the tissue directly. The haemolymph holds the mussels “blood cells” called haemocytes. The haemolymph, together with the haemocytes are involved in several functions, such as; repair mechanism, transport, nutrient digestion, metabolic waste (Cheng 1981), leaving the haemocytes prone to potential toxic effects. These cells can therefore provide information on the health of individuals or populations

(Gustafson et al. 2005). In addition, they react with foreign substances by phagocytosis and is believed to be one of the most important defence mechanisms in bivalves (Hill and Welsh 1966; Cheng 1981).

As haemocytes were used for the investigation of DNA damage, the gills were found to be the most appropriate tissue for assessing alteration in gene expression. The gills are exposed to large volumes of seawater compared to other tissues, making them highly disposed to toxic substances. Furthermore, they are easy to isolate and relatively homogenous in regard to cell type (Dixon et al. 1982; Venier and Canova 1996) and can readily be prepared to a single-cell suspension (White 1937).

1.3 Test compounds

1.3.1 PFOA

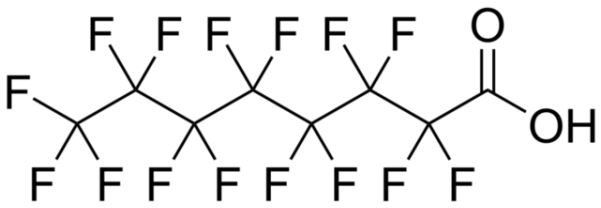

Figure1.1 Structure formula of PFOA.

Perfluorooctanoic acid (PFOA) belongs to a group of chemicals called perfluorinated compounds (PFCs), and like other PFCs it is environmentally persistent, globally distributed, bioaccumulative and possibly harmful. The potential adverse effects of PFC on marine invertebrates have hardly been addressed, and there is still a lack of toxicity data for a comprehensive ecotoxicological valuation of these contaminants (Liu et al. 2013). Their carbon-fluorine bond (C-F) gives them their many unique properties, such as; being resistant to photolysis, hydrolysis, microbial degradation and low metabolic breakdown (Giesy and Kannan 2002). PFOA is one of the most commonly used PFCs and have recently received much attention due to their wide distribution in the environment (Kissa 2001).

PFCs are produced through anthropogenic processes and have been synthesized since the late 1940s. Due to their water- and fat-repellent properties, PFOA and other PFCs have been applied in a range of

consumer and commercial products, including being a residual in the industrial production of fluoropolymers such as polytetrafluoroethylene (PTFE, Teflon® or similar), and can be released from these processes as aqueous or gaseous emission. Some of the products they are used or formed in, as degradation products, are; surfactants, non-sticking cookware, coated food-contact paper (e.g. microwave popcorn bags), protective coating in textiles (e.g. rain gear), lubricants and fire-fighting foams (Kissa et al. 2001; Begley et al. 2005). Exposure to PFOA can be due to releases of PFOA itself, but there also exist a considerable number of precursors, which can lead to PFOA exposure in the environment.

PFOA is highly soluble in water (3.5 g/L) and has a relatively low vapour pressure (2.2 Pa at 20°C), and therefore the aquatic environment is expected to be the primary sink with some additional partitioning to sediment (Environmental Canada 2012). Since the ocean is the major sink of PFCs, marine animals are vulnerable of becoming exposed to their harmful effects. PFOA has been detected in biotic and abiotic samples worldwide, including remote areas, such as the Arctic (Giesy and Kannan 2001; Kannan et al. 2004; Martin et al. 2004a; Martin et al. 2004b; Kannan et al. 2005). It has been shown that PFOA was able to generate ROS in human HepG2 cells (Ericksen et al. 2010). ROS can directly interact with cell organelles or DNA molecules, leading to modifications and possible damage (Liu et al. 2013). Another study on hamster lung cells indicated that related PFCs may increase the genotoxicity of other chemicals (Jernbro et al. 2007). A research on Japanese medaka suggested that PFOA may induce peroxisomal fatty acid oxidation and impose oxidative stress by altering the cellular oxidative homeostasis in the liver (Yang 2010). Other studies show chemosensitivity and endocrine disruption in different organisms such as; mussels (Stevenson et al. 2006), dolphins, (Peden-Adams et al. 2004) and fish (Wei et al. 2007) exposed to PFOA.

The major PFC-producing companies in the world, in conjunction with the US Environmental Protection Agency (EPA), established the PFOA Stewardship Program in 2006. Their goal is to eliminate emissions and product content of these chemicals by 2015 (US EPA 2012). The authorities in Norway have decided to ban the use of PFOA by 1st June 2014 in all consumer products (Norwegian Environment Agency 2013).

1.3.2 Fluoranthene

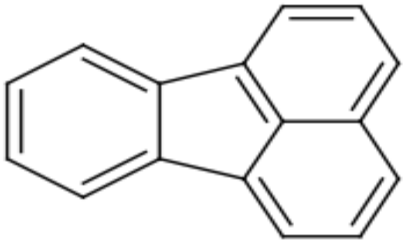

Figure 1.2 Structural formula of fluoranthene.

Fluoranthene is a member of the polycyclic aromatic hydrocarbon (PAH) group of organic compounds. PAHs are an extensive class of hydrophobic contaminants found throughout the marine environment, where anthropogenic activities are the major source of input (NRCC 1983), though natural sources such as volcanic eruption (Ilnitsky et al. 1975) and forest fires (Jenkins et al. 1996) also contributes to their release into the environment). Due to their hydrophobicity (log $K_{ow} > 4$) and high lipophilicity, PAHs in water are bound to suspended particles and accumulate in bottom sediments (Varanasi et al. 1989), with subsequent accumulation in tissues and organs of a wide range of marine organisms (Fabbri et al. 2006).

There are two classes of PAHs; pyrogenic and petrogenic. Fluoranthene is mainly petrogenic, i.e. derived from oil, and is naturally present in fossil fuels (Neff 1979). Some sources of fluoranthene are crude oil, coal tar, motor oil (Verschueren 1983), tobacco smoke (Hoffmann et al. 1972) and a wide variety of cuisine, like charbroiled food (Larsson et al. 1983), and smoked fish (Grimmer and Bohnke 1975). Direct sources of PAHs in the marine environment include sewage, oil spills and runoff (National Research council 1985).

PAHs taken up by an organism may be subjected to biotransformation by enzymatic processes in order to produce more water-soluble compounds, facilitating biliary and urinary excretion (Neff 1985; Varanasi et al. 1989). During biotransformation, certain metabolites are produced, which are known to cause the toxicity associated with PAHs, such as genotoxicity (Akcha et al. 2003b; Wessel et al. 2012). In bivalve mollusks, it is thought that metabolism of PAHs mainly occurs through radical oxidation involving ROS (Stegeman 1985; Winston et al. 1988). It is well known that PAHs cause DNA damage through the

production of DNA SBs and DNA adducts (Nacci et al. 1996; Steinert et al. 1996; Lyons et al. 1999; Aas et al. 2000) and can lead to oxidative stress in marine animals (Hannam et al. 2010; Wessel et al 2010).

Fluoranthene has been reported to be phototoxic, mutagenic and potentially carcinogenic (Kaden et al. 1979; Busby et al. 1984; Kagan et al. 1985; Bos 1987; Tuveson et al. 1987). It is found to be one of the most abundant PAH in the marine sediment (Shiaris and Jambardsweet 1986; Baumard et al. 1998). Nevertheless, there is a paucity of literature concerning fluoranthene biotransformation pathways and its genotoxic effects upon marine organisms (Wessel 2012). The most studied PAH is benzo[a]pyrene (BaP), which is known to be genotoxic in marine organisms (Varanasi and Gmur 1980; Bihari et al. 1990; Venier and Canova 1996; White 2002; Akcha et al. 2003a). Due to a lack of studies on its potential to react with the DNA, fluoranthene is presumed to be less genotoxic than BaP. This understanding can also be expressed due to the presumed lower affinity of fluoranthene to the Aryl hydrocarbon receptor (AHR) (Machala et al. 2001). This receptor plays an important part in the regulation of genes coding for the enzymes involved in the biotransformation process. BaP appears to be metabolised predominantly to quinones in mussel rather than diols as in vertebrates (Sjolin and Livingstone 1997) and relative to mechanisms of toxicity, quinones undergo redox cycling with production of $O_2^{\bullet-}$ making oxidative stress a prominent feature of PAH toxicity in mussel. Brown et al. (2006) found that transcripts isolated from *M.edulis* would during metabolism of BaP, produce an abundance of oxyradicals as many of the cDNAs represented proteins involved either in oxidative stress defence mechanisms or in redox control of signal transduction and cellular signalling pathways.

The toxicity and widespread environmental presence of fluoranthene have placed the compound on the list of the European Water Framework Directive (2000/60/CE) (EU 2000) as one of 33 priority pollutants and one of the 16 priority monitored PAHs of the United States Environmental Protection Agency (US EPA 2009).

1.4 Aim

The overall objective of this study was to evaluate effects of PFOA and fluoranthene, both singly and in combination, on blue mussel, *Mytilus edulis*.

Specific objectives were to:

- Quantify changes in DNA damage in haemocytes

- Quantify the contribution of oxidative stress to observed DNA damage in haemocytes

- Clarify the effects on the expression of selected genes involved in gene ral stress and oxidative stress.

2. Material and methods

2.1 Experimental setup

The exposure system was set at NIVAs Marine Research Station, at Solbergstrand in Drøbak. The exposure and sampling took place between March 2012 until May 2012. The experiment comprised 16 1000-liter flow-through aquarium tanks, with 500 liter seawater in each tank. The aquaria were separated into four different exposure groups, three treatment groups and one vehicle control group. Each of the four exposure groups had four replicate tanks. The seawater was pumped from the fjord at 60 meter depth, into a main header tank used for sedimentation of particles in inlet water. From the header tank, the water was distributed, by gravity, into four secondary tanks, one for each exposure group, through plastic tubes at a rate of 2.43 L/min, giving a flow of 3500 liter water per day.

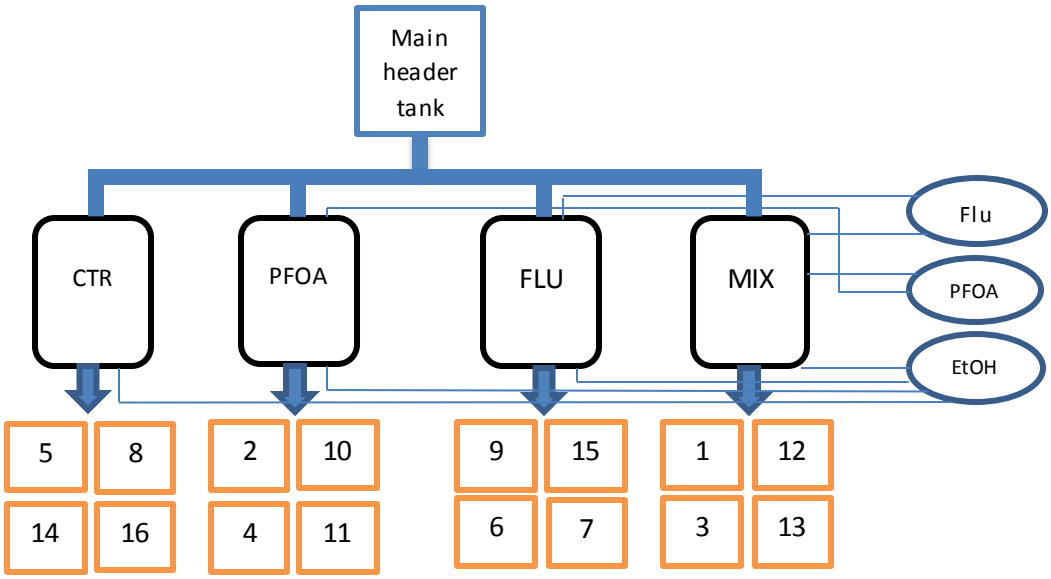

Figure 2.1 The experimental setup. Seawater was pumped into a main header tank and then into four secondary header tanks, where seawater and stock solution were mixed before distributed to the aquariums. There were four treatment groups: vehicle control (tank 5, 8, 14, 16), Perfluorooctanoic acid (PFOA) (tank 2, 4, 10, 11), fluoranthene (tank 6, 7, 9, 15) and mix of fluoranthene and PFOA (tank 1, 3, 12, 13).

The stock solution of the chemicals was diluted in glass tanks with a 1:1 mixture, containing distilled water and ethanol, before pumped to the secondary header tanks. The pump flow from the stock solution to the header tanks was 0.097 ml/min. Plastic tubes to the aquaria connected the secondary header tanks. The seawater in the aquariums was changed four times per day to ensure that the oxygen level were adequate. The water flow from the secondary tanks into the aquariums was therefore 1.4 liter per minute, giving a flow through of 2000 liter water per day, per tank. To keep a constant water level the tanks were drained through a hole in the bottom. Pumps were not required since the header tanks were placed one meter above the aquariums. All of the aquariums were covered with a net lid, in addition to a transparent plastic between each tank to reduce carryover contamination of volatile components (figure 2.2).

Figure2.2 Setup of the tanks showing; the main header tank, secondary tanks, aquarium tanks and the shielding plastic between the tanks.

The mussels were collected at a clean site close to Drøbak, February 2012. Mussels of both sex and similar sizes (50-70 mm) were collected. About one week before the exposure started the mussels were distributed to the aquariums to be acclimatized to their new environment. Ten mussels were kept in small cages hanging from the side of each tank, approximately one meter from the surface. These cages were 50 cm long and 30 cm high.

2.2 Exposure

The treatment groups were exposed to fluoranthene; 5 µg/L, Perfluorooctanoic acid (PFOA); 30 µg/L, and a mix of fluoranthene; 2.5 µg/L and PFOA; 15 µg/L. The experimental concentrations were chosen

based on data from the literature (Oakes et al. 2004; Kirchgeorg et al. 2010; Mhadhbi et al. 2010). The vehicle control received 10 µg/L solvent carrier (ethanol), which was well below the maximum concentration recommended, when using ethanol as a carrier solvent (Hutchinson et al. 2006). The exposure ran for a total of 8 weeks. The staff at Solbergstrand Marine experimental station performed maintenance and daily routines.

2.3 Sampling

Samples were taken four times during the 8 weeks of exposure, which were after 4, 8, 16 and 64 days. The unexposed mussels (0 days of exposure) were kept in a separate tank during the whole experiment and was sampled after the last day of exposure.

Sampling was performed on one individual per aquarium apart from sampling day three, where four mussels were sampled per tank. For the 0-time control, 32 unexposed mussels were sampled. All mussels were measured before the sampling of haemocytes and gill cells.

2.3.1 Isolation of haemocytes and gill cells

Subsequent processing of haemolymph and gills during the sampling took place as follows. First, the valves of the mussels were forced open with a scalpel and the mantel cavity was drained from seawater. This ensured that only haemolymph and not seawater was extracted from the mussels. A volume between 0.2 and 0.5 ml haemolymph was extracted from the anterior adductor muscle by forcing the needle between the two valves. This was performed using a 1 ml sterile syringe that was treated with PBS + EDTA. The cells were resuspended in 0.5 ml PBS + EDTA with a concentration of approximately one million cells per ml and kept on ice. The cell concentration was quantified microscopically for each sample. This was done immediately after extraction, and the cells were counted using a Bürker-Türk haemocytometer with a lens magnification of 10x.

For the extraction of the gills, the anterior muscle was cut with a scalpel to open the shell. The gill on one side was dissected out, put in cryotubes and frozen in liquid nitrogen (-196°C). The samples were later stored at -80°C.

2.4 Comet assay

The comet assay, also called single cell gel electrophoresis (SCGE), is a simple, effective and low-cost technique for measuring DNA damage. The method was first established by Östling and Johanson (1984)

and has since then been used for the detection of DNA damage in single cells. In this study, the protocol from Singh at al. (1998) was used, with some modifications by Tice et al. (2000), Azqueta et al. (2011) and Gutzkow et al. 2013).

Unlike the neutral version (pH 10 during electrophoresis) of the assay, which only detects double strand breaks, the alkali version (pH 13 during electrophoresis) have been used to detect single strand breaks, double strand breaks, and alkali-labile sites (Singh et al. 1988). Cells exposed to genotoxic agents are embedded in an agarose gel and cast on a Gelbond ®film (124 x 58 mm). The film is then incubated in high salt lysis buffer for the extraction of DNA. During the lysis the cellular material and proteins are removed, leaving only the DNA (nucleoids) left in the gel (Cook et al. 1976; Collins et al. 2004). Since the DNA in the cell is wound around a histone core, it becomes negatively supercoiled. Due to the lysis buffer, the histones disengage, while the DNA is still intact and supercoiled. During electrophoresis an electric field is applied, which cause the DNA strand breaks (SBs) to spill out, creating a tail from the nucleus, while the intact DNA remains in the nucleus, giving the appearance of a comet. This happens because damaged DNA relaxes the supercoiling and during electrophoresis the relaxed loops, which is negatively charged, will travel toward the anode (positively charged) in the electrophoresis chamber (Collins 2008). The intensity of the comet tail represents the amount of DNA damage in a cell.

Exposure to certain chemicals can cause elevated levels of oxidative DNA damage (Halliwell and Gutteridge 1999). Implementing specific bacterial repair endonuclease can recognize particular DNA damages. In this study, the cells were incubated with Formamidopyrimidine DNA glycosylase (FPG) to evaluate if PFOA and fluoranthene could lead to oxidative DNA damage in haemocytes of M.edulis. FPG is a DNA glycosylase, which repairs oxidized bases by removing the oxidized base and cuts the sugar-phosphate backbone of the damaged DNA strand. By treating the DNA with FPG during the procedure, additional SBs are created at the site of oxidized bases. These breaks are then detected as an increase in DNA migration within the assay (Gielazyn et al. 2003). FPG recognize DNA damaged purine bases, such as oxidized purines, (8-oxoG), and various ring open purines. (Dusinska and Collins 1996).

The first step in processing of the diluted haemolymph was conducted at the marine station in Solbergstand. It involved suspending the haemolymph in agarose, thereafter casting gels on Gelbond® films, before they were put in lysis buffer for five days. The lysis was followed by electrophoresis,

conducted at the Norwegian Institute of Public Health. The last step, staining and scoring of the films, took place at the University of Oslo.

All work was performed under dim light.

2.4.1 Lysis of the cell

75 mg LMP Agarose (0.75%) was dissolved in 10 ml PBS + EDTA by heating the solution to the boil in a glass beaker. This was performed on a heating plate until the solution was transparent. 225 µl agarose gel was aliquoted in Eppendorf tubes and kept fluid at approximately 37°C using a heating block. 25 µl of the initially diluted haemolymph was then suspended in the aliquotted agarose solution and mixed thoroughly. From this cell suspension, 25 µl was immediately cast in wells on Gelbond® films arranged on pre-chilled aluminium plates (>4 °C), producing eight gels per film. The films had three replicates; one designated for treatment with enzyme, the second as an enzyme reference (buffer incubation without enzyme) and the third as a control (incubation in electrophoresis buffer only). After casting, the films were left to air-dry for 2 minutes, before they were placed in airtight boxes of 50 ml cold lysis buffer for five days.

2.4.2 Enzyme treatment

The Gelbond® films designated for treatment with enzyme and enzyme reference (see section 2.4.1) were immersed in cold Collins buffer (see appendix A) for one hour at 4°C. The Collins buffer was treated with bovine serum albumin (BSA). The films were then replaced with fresh, warm enzyme reaction buffer (0.5 ml BSA per 50 ml Collins buffer). The films for enzyme treatment were treated with FPG. The FPG extract was thawed, and 5 µl was diluted in 45 µl enzyme reaction buffer. The solution was further diluted by transferring 10 µl into 40 µl fresh, warm enzyme reaction buffer. From this solution, 13µl was pipetted into 250 ml (50 ml per film) warm enzyme reaction buffer. This was then distributed to the tubs, containing the films for enzyme treatment. Both the enzyme and enzyme control films were incubated at 37°C for one hour.

2.4.3 Electrophoresis

The electrophoresis working solution buffer was made fresh and refrigerated before use. Unwinding of the DNA molecule was carried out for 20 minutes (1 x 5min, 1 x 15 min) in electrophoresis buffer (pH 13.2) at 4°C. This was performed by placing the Gelbond® films in small tubs containing the buffer. The films were then transferred to the electrophoresis chamber, containing 1.4 liter of fresh buffer. The electrophoresis was performed at 4°C for 20 minutes, applying an electric field of 25 V and 0.8 A. After

electrophoresis, the films were rinsed in neutralizing buffer, first for 5 minutes, then 10 minutes in fresh buffer. This was to prevent further unwinding. Afterwards the films were briefly washed in dH_2O. Finally the films were fixed in 96% ethanol for 5 minutes and then for 90 minutes in fresh 96% ethanol, before they were dried and stored dark until scoring.

2.4.4 Staining

Visualization of the comets was made possible by staining the Gelbond® films with the fluorescent gel, SYBR® Gold. The fluorescent binds to both ss- and dsDNA as well as RNA, and results in the emission of fluorescence. It is thereby possible to visualize the DNA by fluorescence microscopy. There are several dyes available; SYBR® Gold was used as it is found to detect DNA and RNA with greater sensitivity than other stains (Tuma et al. 1999).

The films were stained at room temperature (RT), in the dark for 20 min. This was done in a plastic box, containing 50 ml TE-buffer and 40 µl SYBR® Gold (pre-diluted 10x in DMSO). The box was placed on a rocking table, thereby ensuring that all the gels were stained evenly. After treatment, the films were washed, in distilled water, to remove redundant SYBR® Gold, before it was left to air-dry.

Prior to scoring, the films were placed on a plexiglass plate, and a drop of distilled water was added, both to the film and the plexiglass, before covering the films with cover slides.

2.4.5 Scoring

The visualization of the comets were done by a BX51 microscope from Olympus, through a 20x objective. Attached to the microscope was a mercury lamp that illuminated the stained nucleoids. The microscope was joined to A312f camera that was connected to a computerized image analysis software ("Comet Assay IV" from Perceptive Instruments). This software was used for the scoring of the comets. The scoring was done visually due to a high concentration of cells in some of the gels, which can lead to cells overlapping, thereby making it difficult for the software to discriminate between them. Fifty comets were scored per gel. For each comet the program calculates total intensity (amount of DNA) of the comet, tail intensity (% tail DNA) and head intensity (% head DNA), before calculating percent DNA in the tail versus that of the entire comet. The percent tail intensity is believed to be the best parameter for visual scoring and a good indicator for DNA damage as the tail intensity increases linearly with break frequency (Collins 2004). It was therefore used as a parameter for DNA damage. In addition to overlapping cells, other cells not scored were; abnormal cells (tail in the wrong direction, abnormal shape of nucleoid), cells close to foreign objects and cells close to the edge of the gel. The comets were

scored in a systematic, but random order to avoid double scoring and to ensure that the selected cells represented the whole gel (Collins 2004).

2.5 Gene expression

Real-time quantitative PCR is often the method of choice for quantification of mRNA as it has a high sensitivity, in addition to being comparatively inexpensive (Bustin et al. 2005). Like other PCR techniques, it is based on a multicyclic amplification of the DNA. During an extensive time of the cycle, the amplification of the template DNA transpires exponentially. To be able to quantify the gene expression, the quantification happens during this exponential face. RT-qPCR does this by detecting and measuring products generated during each cycle of the PCR procedure. The quantification happens when the amount of product generated reach a threshold value (Ct-value). The products generated are directly proportional to the input of mRNA (through cDNA) template at the beginning of the PCR process. The technique uses a fluorescent dye, where the increase in dye is related to the quantity of product produced from each PCR cycle. SYBR Green was used in this study since it binds to the dsDNA helix as it is formed. The accumulation of products during the PCR process can be divided into three phases (Figure 2.2). During the first phase, called the baseline, the level of product increases, but the fluorescent signal is still too low to be detected by the instrument (Arya et al. 2005). The fluorescent emission from the product at each time, minus the fluorescent emission of the baseline, called ΔRn, is plotted against the number of cycle. A threshold is an arbitrary level of fluorescence calculated based on the baseline, where signals above the threshold can be used to define the threshold cycle (Ct) for a sample. The Ct value is defined as the amount of cycles needed before reporter fluorescent is higher than the threshold (Heidi et al. 1996; Gibson et al. 1996). The more mRNA template prior to the start of the qPCR reaction, the fewer cycles are needed to accumulate enough product to reach the Ct value (Gibson et al. 1996). Above the threshold the DNA amplification increases exponentially. As reaction components becomes limiting, the rate of target amplification declines until the plateau phase is reached, and there is little or no increase in PCR product (Arya et al. 2005).

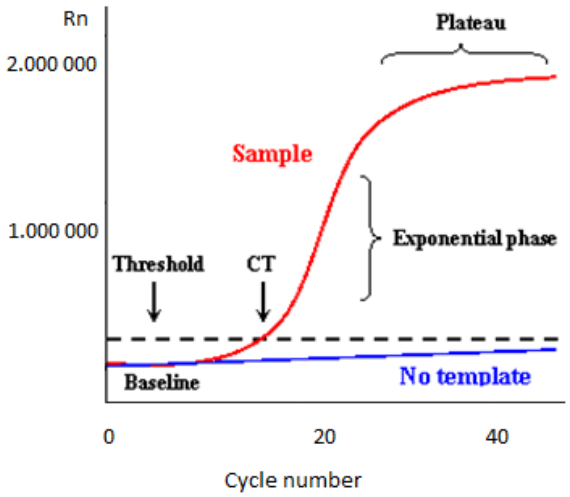

Figure2.2 Amplification of cDNA. The product generated during the process is not detected before it reaches the threshold line. The Ct value is the number of PCR cycles when the threshold is reached. From this point the amount of cDNA increases exponentially, until the amplification declines and the process reaches the plateau phase. Modified from Arya et al. (2005).

Reference genes

During an experiment there are a number of errors that can occur. These errors could be different input of RNA amount, or differences in efficiency of cDNA synthesis and PCR amplification. Errors like this can be reduced by simultaneously amplifying cellular RNA with the target. These RNAs will serve as an internal reference against which other RNA values can be normalized. The genes, called reference genes or housekeeping genes, should be expressed at a constant level within different tissues at all stages of development and have the same constant level of expression under different experimental conditions (Arya et al. 2005). In this study, two reference genes were used where the mean expression of these genes were used for the normalization as recommended by Vandesompele et al. (2002).

The analysis of gene expression in *Mytilus edulis* was investigated on gill tissue. The harvesting of the tissue is explained in section 2.3.1. Before the amplification of the RNA (through cDNA), the RNA has to be isolated from the cell. After the isolation, the RNA was converted to complementary DNA (cDNA), which is implemented with a DNA-binding dye for the detection of the cDNA during the qPCR process.

The following procedures were conducted at the University of Oslo.

2.5.1 Homogenization of tissue

Approximately 30 mg gill tissue was transferred into 2 ml Precellys-tubes containing sterile crushing beads covering the bottom of the tubes, in addition to 0.5 ml trizole. Precellys® 24 homogenized the samples with a frequency of 15000 rpm, 3x15 seconds. A cooling device, Cryolys, prevented the samples from overheating due to the mechanical impact between beads and tissue during motions. The Cryolys sprayed cold air (-50°C) around the tubes in the Precellys, so that the temperature inside the tubes remained at approximately 4°C. The Cryolys was filled with circa 2 liters of liquid nitrogen before each run. After homogenization, the homogenate was centrifuged at 10.000 g for 1 minute at 4°C, to ensure that the cell debris was beneath the crushing beds. Subsequently the homogenate was incubated on ice for 5 minutes.

2.5.2 RNA isolation

In addition to mRNA, miRNA from day 0 and day 3 was isolated, as miRNA from these samples was to be used in another study. There was therefore performed two procedures for the isolation of RNA.

Trizol method

0.1 ml chloroform was added to the homogenate to separate the samples into three phases; a lower organic phase, an interphase and an upper aqueous phase. The samples were shaken vigorously by hand for 15 seconds, before incubated for 2-3 minutes on ice. The samples were then centrifuged at 12 000 g for 15 minute at 4°C. After centrifugation, the upper colourless aqua phase, which contained the RNA, was extracted and aliquoted to eppendorf tubes. Approximately 50-150 µl from each sample.

0.2 ml isopropanol was added to the sample for RNA precipitation. The samples were mixed well and incubated on ice for 10 minutes, before it was centrifuged at 12 000 g for 15 minutes at 4°C. The supernatant was removed, and the RNA pellet was washed with 0.5 ml 75% ethanol and vortexed for 20 seconds. The samples were then centrifuged at 7500 g for 5 minutes at 4°C. All of the ethanol was removed, and the pellet was left to air-dry for 5-10 minutes at RT. The pellet was re-dissolved in 100 µl RNase-free water and incubated for 10 minutes at 58°C. The samples were then stored at -80°C.

mirVana method

The mirVana miRNA isolation kit (Ambion®) from life technologies was used for the isolation of both total RNA and small RNA from the same sample. All the centrifuging steps were performed at RT.

To extract the total RNA, 3.75 µl of "Homogenate Additive" was added to the homogenate and incubated on ice for 10 minutes. 300 µl of Acid-Phenol: Chloroform was supplemented and vortexed for

30 seconds. The homogenate was centrifuged at 10 000 g for 5 minutes to separate the organic and aqueous phase. After the centrifugation, 300 µl of the aqueous (upper) phase was extracted and aliquoted into collection tubes provided with the kit. 100 µl of 100% ethanol was added to the aqueous phase in the collection tubes and vortexed. The samples were then aliquoted to a filter, placed onto new collection tubes, and centrifuged at 10 000 g for 15 seconds. To extract the total RNA the filter was treated with 700 µl "RNA Wash Solution 1" and centrifuged for 10 seconds. The flow-through was discarded, and the filter was treated with 500 µl "Wash Solution 2/3" and centrifuged as the previous wash. The step with "Wash Solution 2/3" was then repeated, and flow-through was discarded. The filter was then spun for 1 minute to remove the residual fluid from the filter. To recover the RNA, the filter was treated with 100 µl pre-heated (95°C) nuclease-free water and centrifuged for 30 seconds. The eluate was collected and stored at -80°C.

2.5.3 RNA quantity measurement

The concentration and purity of RNA in each sample was determined by spectrophotometric measurement using a microplate reader that measured the optical density (OD). The microplate measures the absorbance at 260 nm (A260), the concentration of RNA in µg/µl and the purity of the sample (260/280 ratio). The microplate reader SynergyMx MultiMode was used for the measurement combined with Gen5 data analysis software. No pretreatment of the samples was necessary. A blank sample of RNase-free water was used to reset the instrument, before 2 µl of each sample was quantified.

The purity of RNA in a sample was tested by quantifying the wavelength ratio between RNA and protein. RNA absorbs light at an absorption maximum of 260 nm, while protein has an absorption maximum of 280 nm. The ratio should be between 1.8 and 2. Equivalently the ratio between absorbance at 260 and 230 nm was used to evaluate the contamination from compounds containing peptide bonds or phenol rings, which could inhibit enzymatic reactions (Gallagher and Desjardins 2008).

2.5.4 RNA quality measurement

The quality of the RNA was tested by Agilent 2100 Bioanalyzer, from Agilent. The bioanalyzer use gel electrophoresis to quantify the fragmentation of RNA by measuring 18S and 28S ribosomal subunits. All RNA samples were run with the RNA Nano 6000 Kit on RNA Nano chips from Agilent.

Prior to the preparation of the gel a RNA ladder, applied with the kit, was prepared by pipetting the ladder in RNase-free vial. Then the ladder was spun down and denatured for 2 min at 70°C, using a

heating block, before it was cooled on ice. The ladder could then be aliquoted in recommended amounts for daily use and stored at -80°C.

All the reagents were equilibrated to room temperature for 30 minutes before use.

Approximately 550 µl of Agilent RNA 6000 Nano gel matrix was transferred into a spin filter and centrifuged at 1500 g for 10 minutes. Aliquots of 65 µl filtered gel was then placed in RNase-free microtubes. After the gel dye mix had equilibrated to RT, it was vortexed for 10 seconds and spun down. 1 µl of dye was pipetted into RNase free microtubes containing the premade filtered gel. This was then mixed into a homogeneous solution and centrifuged at 13 000 g for 10 minutes at RT (One microtube is enough for one chip).

A microchip was loaded with 350 µl of isopropanol and placed in the bioanalyzer for 1 minute, before adding the gel dye mix. Another microchip was filled with 350 µl RNase-free water and placed in the bioanalyzer for 10 seconds. This was performed to decontaminate the electrodes of the Bioanalyzer. The decontamination was also performed between each run. After the decontamination 9.0 µl gel-dye mix was pipetted to the well marked ^G on a new Agilent RNA Nano chip. The chip was placed on a chip priming station. The priming station was closed, and pressure was added to the chip for 30 seconds by a plunger, thereby distributing the gel-dye mix over the whole surface of the chip. Then, another 9.0 µl of gel dye mix was pipetted to the well marked G. After the gel-dye mix was added 5 µl of the RNA 6000 Nano marker was pipetted into the well, marked with a ladder symbol and each of the sample-wells, before 1 µl of the ladder was pipetted to the well marked with a ladder. 1 µl of each sample was pipetted to 12 sample-wells on the chip, which was then vortexed at 2400 rpm for 1 minute. At last, the chip was placed in the bioanalyzer for 30 minutes.

2.5.5 cDNA synthesis

As RNA cannot serve as a template for PCR, the first step in an RT-qPCR assay, after isolation of RNA, is a reverse transcription of the RNA template into cDNA, performed by the enzyme reverse transcriptase (Bustin 2000). The kit Transcriptor first strand cDNA synthesis kit from Roche was used, which includes the enzyme AMV reverse transcriptase. The reverse transcription of the RNA was performed using the thermo cycler Mastercycler ep Gradient S, from Eppendorf. All reaction had three technical replicates. The reagents were thawed on ice and briefly centrifuged before use.

10 µl of each RNA sample was reversed transcribed on a 96-well PCR plate from thermo scientific. Each sample was treated with 1 µl of a mix of Oligo(dT) primer and 2 µl random hexamer primer. To circumvent secondary structures the 96-well plates, covered with sealing foil to avoid condensation, were placed in the Mastercycler and denaturated at 65°C for 10 minutes. After denaturation the plates were put back on ice, and each template was allocated 7 µl reverse transcription master mix, containing: 4 µl Reverse Transcriptor reaction buffer, 0.5 µl Protector RNase Inhibitor, and 2 µl deoxynucleotide solution mix. The plates were sealed and briefly centrifuged in a centrifuge, containing a rotor for multiwell plates with suitable adaptors, to avoid air bubbles. At last, the plates were incubated in the Mastercycle under following conditions:

- 25 C˚ for 10 minutes for primer annealing
- 55 C˚ for 60 minutes for RT reaction
- 85 C˚ for 5 minutes for transcriptase denaturation.

After incubation, the plates were immediately put on ice to stop further reaction, before stored at -20˚C up until the qPCR step.

2.5.6 Real-Time quantitative Polymerase Chain Reaction

The PCR process happens in three steps, where the amount of DNA-copies doubles for each cycle. The first step is the denaturation of dsDNA, where the DNA strands separates due to high temperature (95°C). The second step is the annealing of the primers to the DNA template. The temperature drops below the melting point (<70°C), allowing the primers to hybridize to the complementary sequence on the ssDNA template. The final step is the elongation of the new complementary DNA. The temperature increases (60-78°C) so that DNA polymerase can start the synthesis of the new strand by copying the DNA template, resulting in two identical DNA strands (Figure 2.3). The cycle then repeats itself by separating the DNA again.

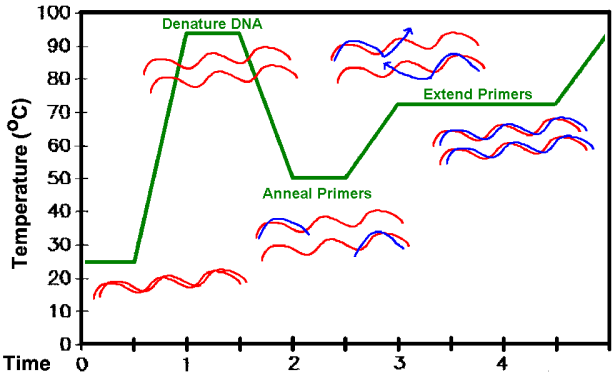

Figure 2.3 The RT-qPCR process. cDNA denaturizes to ssDNA followed by a cooling, which leads to the annealing of the primers to the ssDNA. The temperature then rises again and activates the DNA pol, which then synthesize the new DNA strand. Illustration from Carr (2010).

The qPCR assay for quantification of mRNA expression was performed using the kit SYBR Green I Master mix, and the instrument Lightcycler®480 system was used for the amplification of cDNA, both from Roche

The selection of primers was based on articles on gene expression for bivalves mollusks exposed to contaminants, see table 2.1 (Dondero 2006; Dondero et al. 2006). The primers were purchased from Invitrogen and stored at -20°C. Target genes were selected based on their potential involvement in mechanisms of pollutant and xenobiotic response, including genes involved in general stress and oxidative stress.

Table2.1 Primers used for gene expression analysis.

Genes	**Biological function**	**Genebank number**	**Primers**
28S rRNA (reference gene)	Ribosome	Z29550	F: 5-ACTCGCGCACATGTTAGACTC-3 R: 5-AGCCACTGCTTGCAGTTCTC-3
EF1 (reference gene)	Ribosome	AY580270	F: 5-CACCACGAGTCTCTCCCAGA-3 R: 5-GCTGTCACCACAGACCATTC-3
Mt10	Metallothionein	AY566248	F: 5-GGGCGCCGACTGTAAATGTTC-3 R: 5-CACGTTGAAGGYCCTGTACACC-3
P53	Tumour suppression	DQ158079	F: 5-CCAACTTGCTAAATTTGTTGAAGA-3 R: 5-TTGGTCCTCCTACACATGAC-3
Kinase (Krs)	Stress responsive	DQ158074	F: 5-AGCTACGCTGATGTTGGACA-3 R: 5-AGTTGACCTGCCACACCAAA-3
RNA helicase	RNA structure modification	DQ158075	F: 5-GGCTGTTTGTTCGGTGGATG-3 R: 5-CTGCTCTCACTTGTGAAGGGT-3

Primer efficiency

The amplification efficiency for each primer pair was tested by making a standard curve with dilution series (80 ng/µl, 20 ng/ µl, 5 ng/µl, 1 ng/ µl and 0.3 ng/µl). The standard curve of each primer pair was then used to calculate the amount of amplified cDNA. 10 µl from 12 random cDNA samples that already

had been diluted 3x was mixed in an eppendorf cup and allocated in RNase free water. A master mix was made for each primer pair with components provided with the kit. The master mix for one sample; contained 10 µl SYBR green master mix, 0.5 µl forward primer (10 µM), 0.5 µl reverse primer (10 µM) and 6 µl RNase-free water. This was prepared in an eppendorf cup under dim lights to avoid bleaching of the fluorescent dye. 2 µl of the standard curve and 8 µl master mix were then allocated to a 96-well plate (LightCycler® 480 Multiwell Plate 96) from Roche, in triplicates. Negative template control was applied for each primer pair, where the samples from the standard curves were replaced with nuclease free water, thereby controlling the samples for primer-dimers or potential contaminations. The plates were sealed with sealing foil (LightCycler® 480 Multiwell Sealing Foil), and centrifuged at 1500 g for 2 minutes to remove air bubbles and to spin down the content. The plates were then placed in the Lightcycler and run with the program described in table 2.2. The slope of the standard curve, for each primer pair, was used to calculate the amplification efficiencies (E) of the primer. The calculated mean efficiency ranged from 1.9 to 2, meaning that the templates were approximately doubled for each amplification cycle. To identify formation of possible primer-dimers, melting curves were included at the end of each run.

Quantification by RT-qPCR

The mixing of cDNA and master mix was performed using the pipetting robot "Bravo Automated Liquid Handling Platform", from Agilent technologies. This reduced the workload of pipetting. For each gene, a primer specific master mix was prepared in the same way as for the primer efficiency test. Then, 28 µl master mix was subsequently pipetted into the wells of a 96-well plate (LightCycler® 480 Multiwell Plate 96). The cDNA was diluted 3x to a concentration of 330 ng/µl, to ensure that there were enough template for all of the qPCR reactions. The cDNA was then transferred into a new 96-well plate. The plate with the master mix and the plates with the diluted cDNA were then placed in the robot, which allocated 8 µl master mix and 2 µl cDNA from each sample into the same 384-well plate (LightCycler® 480 Multiwell Plate 384). After pipetting, the 384-well plate was sealed with a sealing foil, and centrifuged at 1500 g for 2 minutes. The plate was subsequently located in the Lightcycler and run with the program described in table 2.2. The Ct-value of the fluorescence curve, of the respective gene, could then be calculated by implementing the external standard curve, in the Lightcycler®480 software, made during the primer efficiency test.

Table 2.2 The PCR program consisted of a brief pre-incubation, followed by 45 cycles of amplification, melting curve analysis and finally cooling of the reactions.

Step	Temperature (˚C)	Time (s)
Pre-incubation	95	300
Amplification 45 cycles	95	10
	60	10
	72	10
Melting curve analysis	95	5
	65	60
	97	-
Cooling	40	30

The raw data generated in the Lightcycler® 480 software were exported to Microsoft Excel 2013. Normalization was performed according to the ΔΔCT-method (Livak and Schmittgen 2001), see appendix B, where each gene expression is normalized against a reference gene within each sample. This gives the normalized relative quantification (NRQ), which compensate for the differences between samples. The fold change (relative expression) gives the ratio between the NRQ of the exposed group and NRQ of the control group (unexposed samples and vehicle control). The ratios demonstrate the relative difference in gene expression between groups. The calculation takes into account the efficiency of the primer pair by using the amplification efficiency of each primer. Ideally, the primer efficiency is equal to two, however, since the efficiency can vary between the different primer pairs, using the estimated primer efficiency can therefore give a more precise result (Rieu and Powers 2009).

2.3 Statistical analysis

Statistical analyses of comet data were carried out using the percent tail DNA intensity and parametric tests. Parametric tests such as the analysis of variance (ANOVA) rely on assumptions of independence, normality and equal variances. Normal distribution of data was checked using the Shapiro-Wilkinson test (Shapiro & Wilkinson 1965). All data were checked for homogeneity of variance using Levene's test (Levene 1960). For data with variance equality, treatments were compared using student t-test between two groups and one-way analysis of variance (ANOVA) between more than two groups (Zar 2010). In cases where one-way ANOVA was significant, Tukey-Kramer post hoc test was used to identify where the difference was while Dunnett`s post-hoc test was used to compare treated samples against 0-time and respective vehicle control (Dunnett 1955). If there were significant heterogeneity in the variance, a log

transformation of data was performed. If the variance remained unequal, nonparametric tests were used. Mann-Whitney test was used to compare means between two groups, while Kruskal-Wallis test was used to compare means between more than two groups. In cases where Kruskal-Wallis test was significant, Dunn post-hoc test was used to compare the differences between treated samples against unexposed samples and vehicle controls (Dunn 1964). The data is presented as median and standard deviation. The graphical presentation of data was by box plots with median quartiles and 10% - 90% percentiles (whiskers).

For the analysis of Real-Time qPCR data, the Ct-values from the Lightcycler®480 software were exported to excel, where NRQ of each gene within a sample was calculated. Statistical analysis was performed using non-parametric tests. Mann-Whitney was used for paired comparison of samples, while Kruskal-Wallis was used for comparing more than two groups. The comparison of treatment groups against 0-time and vehicle control was conducted using Dunn post-hoc test. The graphical presentation of data was by box plots with median, quartiles and 10% - 90% percentiles (whiskers).

Statistical analysis of both comet data and qPCR data were carried out using the software JMP 10 (SAS Institute Inc.) and graphically displayed using GraphPad Prism 6 (GraphPad Software, Inc.) . For all results, $p < 0.05$ was considered statistical significant (Cowles and Davis 1982, Zar 2010).

3. Results

3.1 DNA damage

DNA damage in haemocytes exposed to Perfluorooctanoic acid (PFOA), fluoranthene and the combined exposure of these two (mix), was quantified as the proportion of DNA in the comet tail (% tail DNA) after 0, 4, 8, 16 and 64 days of exposure.

The unexposed mussels (0 days of exposure) were sampled prior to spawning, and it was therefore feasible to distinguish the sex of each mussel. This made it possible to investigate if there were any differences in DNA damage between female and male for unexposed samples. Analysis using the Student t-test showed that there were no significant differences in tail DNA intensity between females and males ($p = 0.7$) (Figure 3.1).

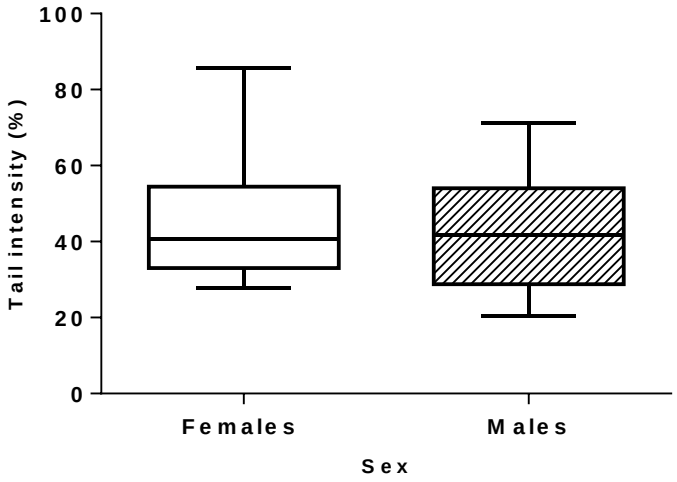

Figure 3.1 DNA tail intensity in unexposed mussels. Data presented as median, quartiles and 10-90 percentiles.

3.1.1 Effect of contaminant exposure

Statistical analysis showed normal distribution and equal variance (Levene, $p>0.05$) of comet data for treatment groups, including the unexposed group, within the same sampling time. Parametric tests were therefore used to investigation if there were any significant differences between exposed and unexposed samples (table3.2).

Table 3.2 p-values of tail DNA in haemocytes between treatments for each of the sampling days. Asterix indicating a statistical difference (P>0.05) compared to unexposed samples (0 days of exposure).

Exposure time (days)	Individuals	p-value
4	16	0.34
8	16	*0.01
16	16	0.19
64	16	0.41

Analysis using one-way ANOVA indicated a significant decrease in tail DNA intensity for mussels exposed to PFOA (Dunnett`s, p= 0.02 n=4) and fluoranthene (Dunnett`s, p= 0.02, n=4) compared to unexposed samples after 8 days of exposure (figure 3.2). None of the mussels exposed for 8 days were significantly different to their corresponding vehicle control (ethanol exposure only) (Dunnett`s, p > 0.05), as indeed did any of the mussels from the other sampling days (p > 0.05).

A comparison between unexposed and vehicle control was also performed, where a significant increase in tail DNA was observed for unexposed samples after 8 days (Tukey-Kramer, p= 0.03) and 16 days (Tukey-Kramer, p= 0.03) of exposure.

Variances for vehicle control between the different days were significantly different, and hence violated one of the assumptions of ANOVA (Levene, p= 0.02). Therefore, non-parametric tests were used, which indicated a significant difference in tail DNA (Kruskal-Wallis, p= 0.03) between mussels sampled on day 16 and 64 (Dunn, p= 0.03 n=4). There was a high variability in tail DNA intensity between individuals after 4 days of exposure (14 – 38%) compared to mussels after 8 days and 16 days of exposure. These mussels showed a similar distribution of variance in tail DNA, thereby showing low inter-individual variability compared to mussels from day 4 (Figure 3.2). Decreased inter-individual differences on day 8 and 16 may have been an indication of adaption to ethanol exposure.

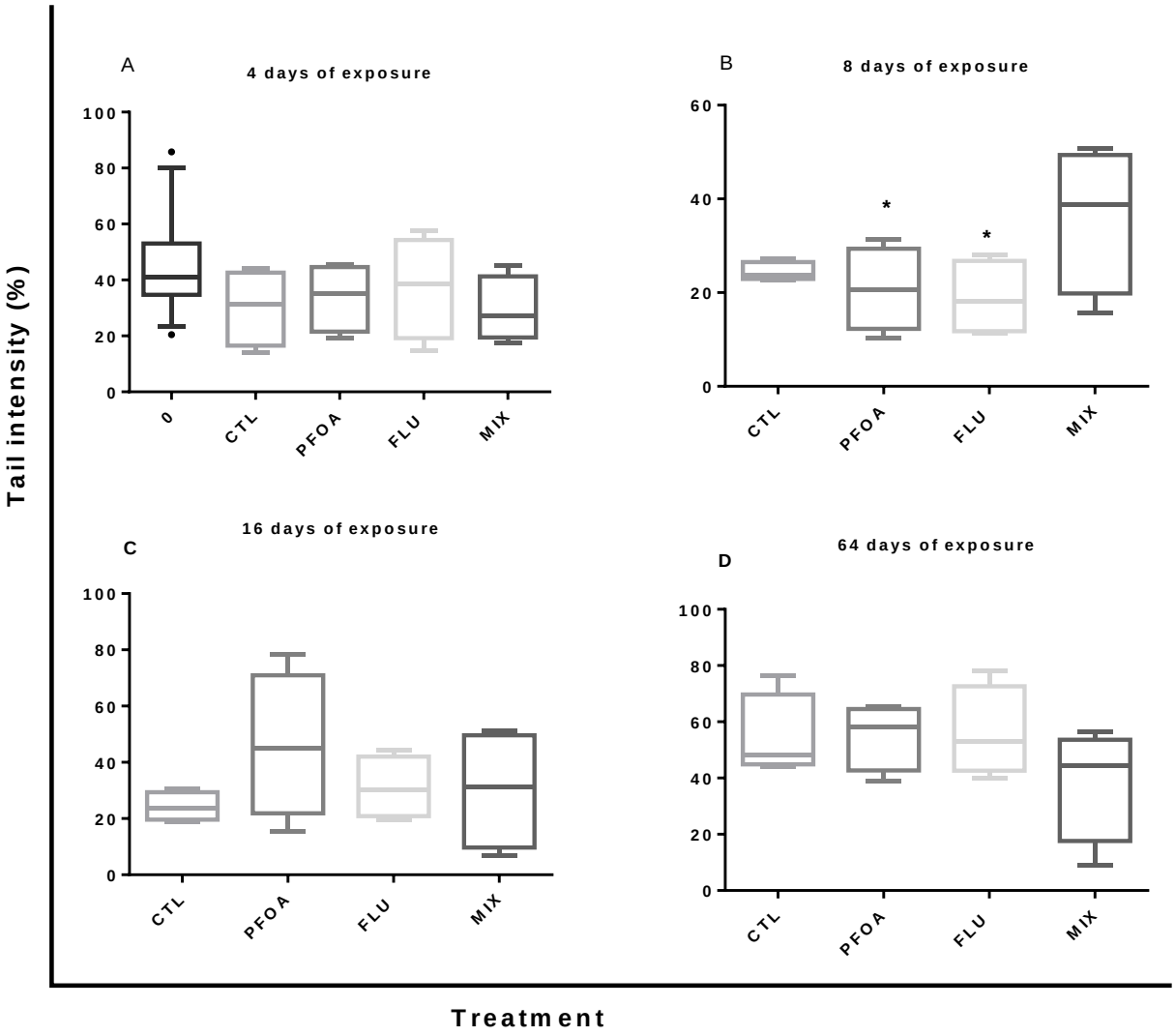

Figure: 3.2 Tail DNA in haemocytes between the four treatment groups at 0, 4, 8, 16 and 64 days of exposure. Data presented as median, quartiles and 10-90 percentiles. Asterisks indicate a statistical difference compared to unexposed samples. A) 4 days, B) 8 days, C) 16 days D) 64 days of exposure. No significant differences were found for any of the treatment groups when compared to their corresponding vehicle control.

3.1.2 Effect of exposure duration

A comparison of the different time points within the same treatment, using one-way ANOVA, indicated a significant difference in tail DNA intensity for samples exposed to fluoranthene ($p=0.01$). Mussels exposed for 64 days had a significant increase in tail intensity (Tukey-Kramer, $p=0.01$, $n=4$) compared to mussels exposed for 8 days. Mussels exposed for 8 days, and 64 days were not significantly different

from their corresponding vehicle controls (Dunnett`s, $p > 0.05$), which was also the case for mussels from the other sampling days (ANOVA, $p > 0.05$).

Mussels exposed to PFOA showed no significant difference in tail DNA between sampling days, using one-way ANOVA ($p = 0.06$), or to their corresponding vehicle control (Dunnett`s, $p > 0.05$, $n = 4$). The same was observed in samples co-exposed to PFOA and fluoranthene between the sampling days ($p = 0.6$), and their corresponding vehicle control (Dunnett`s, $p > 0.05$, $n = 4$).

3.2. Oxidative stress

Treating the cells with the lesion specific enzyme Formamidopyrimidine DNA glycosylase (FPG), was expected to increase the migration of DNA as a quantity of oxidative DNA damage.

Significant increases in oxidative DNA damage were detected in unexposed cells, treated with FPG compared to a reference sample (Student t-test, $p < 0.01$, $n = 16$), indicating that the enzyme did induce FPG sensitive sites (Figure 3.3).

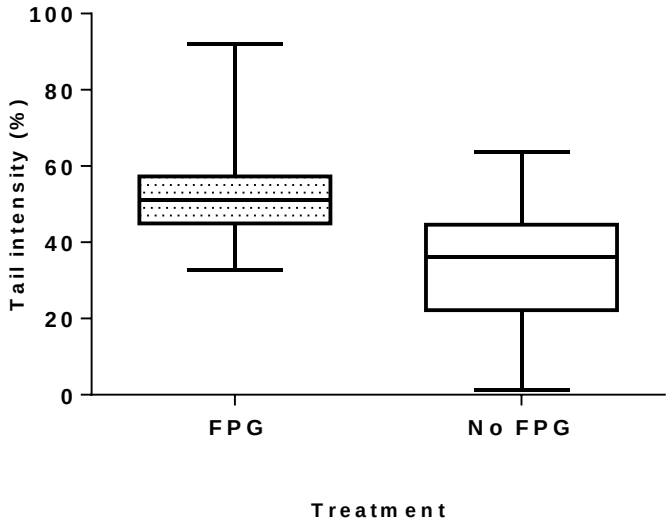

Figure 3.3 Tail intensity of DNA indicated significant differences in oxidative DNA damage between enzyme-treated cells and no enzyme-treated cells in unexposed mussels. Data presented as mean, quartiles and 10 - 90 percentile.

Technical replicates of each sample were incubated with FPG. These results were then derived from the results from the technical replicates that were incubated with the same enzyme buffer, only without the

enzyme (reference samples). This gave the amount of oxidative DNA damage for each mussel within an exposure groups. Comparing exposed groups to the unexposed group showed a significant decrease in oxidative DNA damage after 64 days for mussels exposed to PFOA (Student t-test, p=0.01) and mussels exposed to fluoranthene (Student t-test, p= 0.02). The vehicle control was also compared to the unexposed group and showed a significant reduction in oxidative damage after 4 days of exposure (Student t-test, p= 0.04). Comparing exposed groups to vehicle control showed a significant difference for PFOA-exposed mussel only, where there was an increase in oxidative damage after 16 days. (Student t-test, p= 0.04) (Figure 3.4).

Comparing the duration of the exposures, a significant increased amount of oxidative DNA damage for mussels exposed to PFOA after 16 days, compared to 4 days (Tukey-Kramer, p= 0.02, n= 4), 8 days (Tukey-Kramer, p= 0.02, n= 4) and 64 days (Tukey- Kramer p= 0.01) of exposure. There were no significant differences for mussels exposed to fluoranthene or both PFOA and fluoranthene (mix), between the different time points (Figure 3.4).

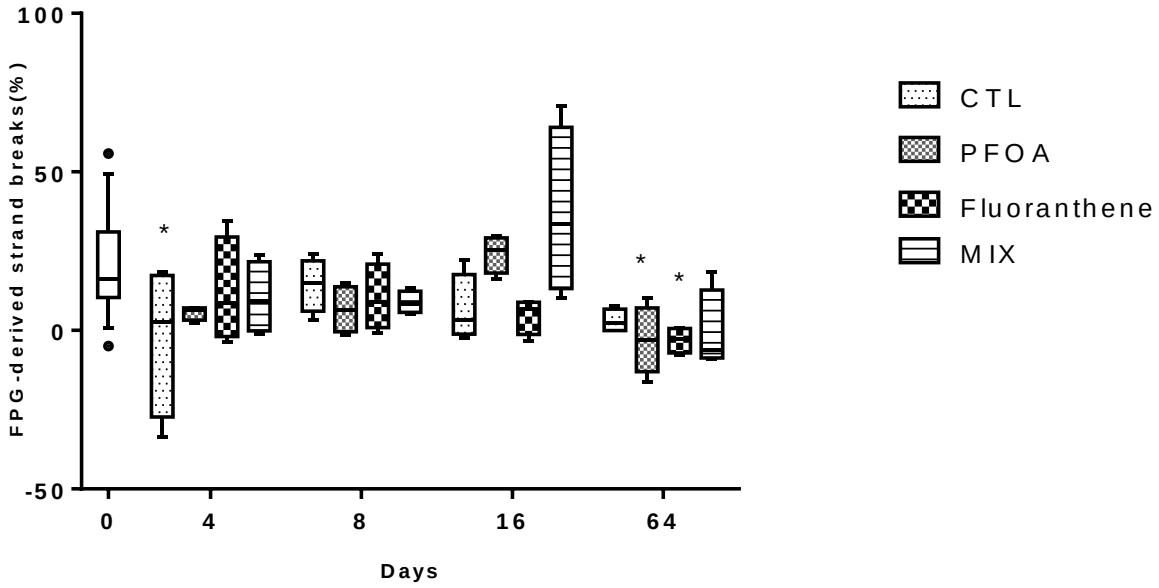

Figure 3.4 FPG-sensitiv (oxidative DNA damage) sites. *significant differences compared to unexposed samples. A significant increase in oxidative damage was found for PFOA-exposed samples compared to vehicle control (CTL), after 16 days of exposure. Data presented as median, quartiles and 10 - 90 percentile.

3.3 Gene expression

The expression of the genes; metallothionein 10 (Mt10), p53, RNA helicase and stress responsive kinase (Krs) was performed on total RNA from gill tissue in mussels exposed for 0, 4, 8 and 16 days, including vehicle control. Expression data of each gene was normalized using Elongation factor-1 (EF-1) and 28s ribosomal RNA (28s rRNA) as internal reference genes. Ratios between exposed groups and unexposed groups were calculated using the normalised values (table 3.1). The ratios demonstrates the relative difference in gene expression between groups. The aim was to evaluate whether single compound exposure and/or coexposure of PFOA and fluoranthene would influence the regulation of genes involved in oxidative stress and general stress.

Table 3.1 fold change ratios for the target genes at three different time points. Ratios between median expression of each gene to the expression level of the unexposed samples (0) and vehicle control (CTL). Asterix indicates significant difference compared to unexposed mussels.

Sample day 4	**Gene**							
	Mt10		***p53***		***RNA Helicase***		***Krs***	
Treatment	Fold change		Fold change		Fold change		Fold change	
	0	CTL	0	CTL	0	CTL	0	CTL
CTL	1.78		1,17		0,98		1,97	
PFOA	1.33	0.75	*2,46	0,00	0,95	0,97	*7,28	3,69
Flu	1.92	1.08	*4,08	3,48	0,94	0,96	3,65	1,85
Mix	0.62	0.35	1,08	0,53	0,96	0,97	1,36	0,69
Sample day 8	**Gene**							
	Mt10		***p53***		***RNA Helicase***		***Krs***	
Treatment	Fold change		Fold change		Fold change		Fold change	
	0	CTL	0	CTL	0	CTL	0	CTL
CTL	7,25		4,17		1,04		5,98	
PFOA	1,72	0,24	0,80	0,19	1,04	1,00	1,75	0,29
Flu	2,77	0,38	1,67	0,4	1,02	0,98	3,65	0,61
Mix	1,66	0,23	1,31	0,31	1,04	0,99	2,00	0,34
Sample day 16	**Gene**							
	Mt10		***p53***		***RNA Helicase***		***Krs***	
Treatment	Fold change		Fold change		Fold change		Fold change	
	0	CTL	0	CTL	0	CTL	0	CTL
CTL	0,53		1,34		1,02		2,35	
PFOA	1,63	3,07	0,48	0,35	0,96	0,94	1,73	0,73
Flu	0,42	0,80	1,29	0,96	1,02	1,06	2,61	1,11
Mix	2,14	4,03	1,27	0,94	0,84	0,82	-	

Median and non-parametric tests were used for the statistical analysis (n=3 or 4). Comparing the expression of genes for exposed groups against unexposed group (0 days of exposure) showed that day 4 was the only sampling time that was significantly altered compared to the unexposed samples. Statistical analysis using Kruskal-Wallis` test showed a significant increase in gene expression of *p53* (P= 0.01) *and Krs* (P=0.04) (Figure3.1). Dunn post-hoc test revealed a significant increase in the expression of *p53* for

samples singly exposed to PFOA (p= 0.02, n= 4) and fluoranthene (P= 0.02, n= 3), and an increase in the expression of *Krs* (p= 0.03, n=4) for PFOA-exposed samples.

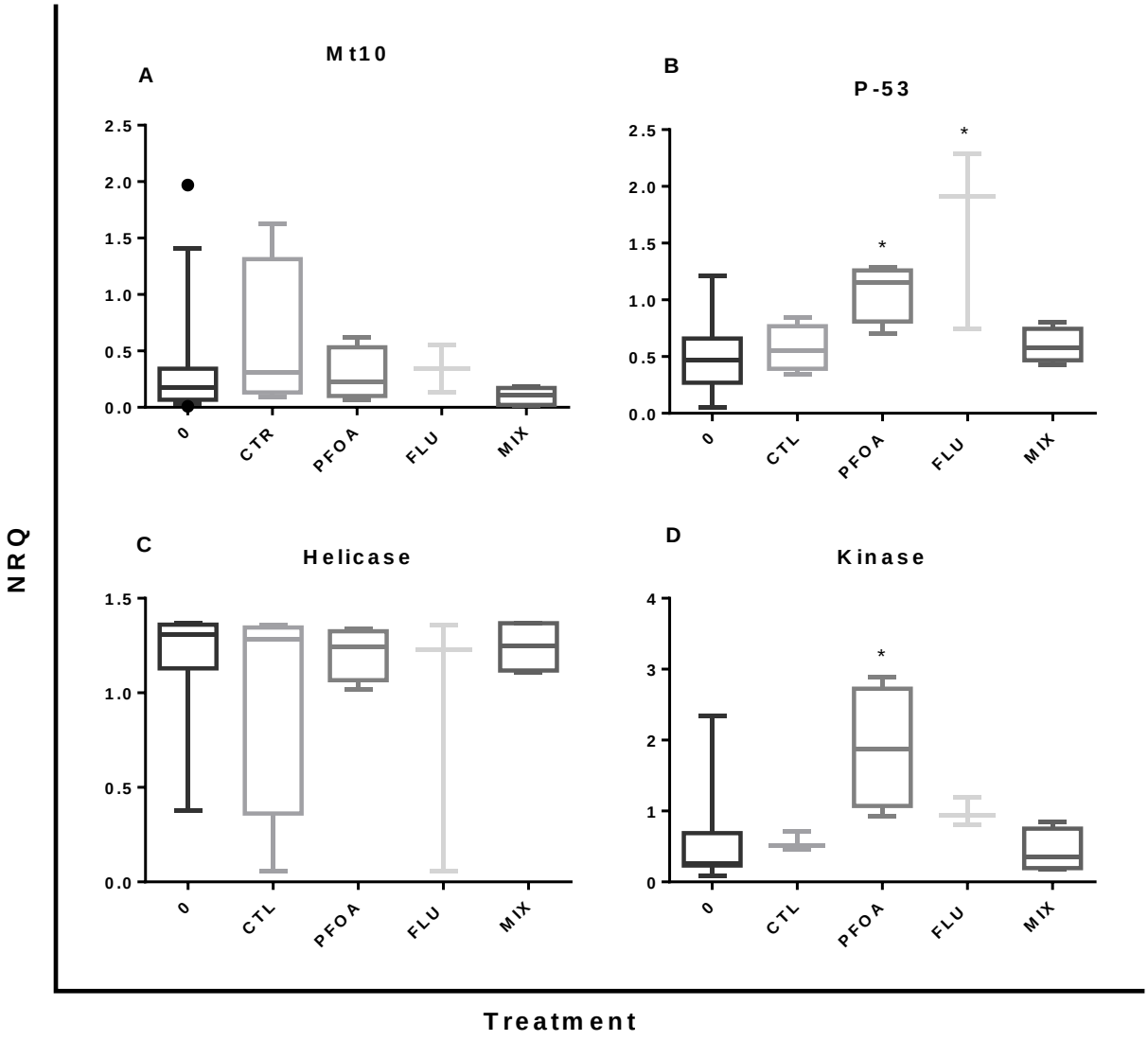

Figure 3.1 Normalized relative quantified gene expression for mussels sampled after 4 days of exposure, where asterisk* indicate a significant difference compared to unexposed samples. Data presented as median, quartiles and 10-90 percentiles. A) expression of *Mt10*. B) expression of *p53*. C) expression of *RNA helicase*. D) expression of Kinase (Krs).

Gene expression levels were also performed for vehicle control compared to unexposed mussels. A significant increase was found in the expression of *Mt10* (Mann-Whitney, p= 0.02, n= 4), after 8 days of exposure. A significant increase was also found in the expression of *p53* for vehicle control, exposed for8 days (Mann-Whitney p=0.02, n= 3) (Figure 3.2). A significant decrease of *p53* was observed for PFOA-exposed samples compared to vehicle control (Mann-Whitney, p= 0.05, n= 3), also after 8 days of exposure.

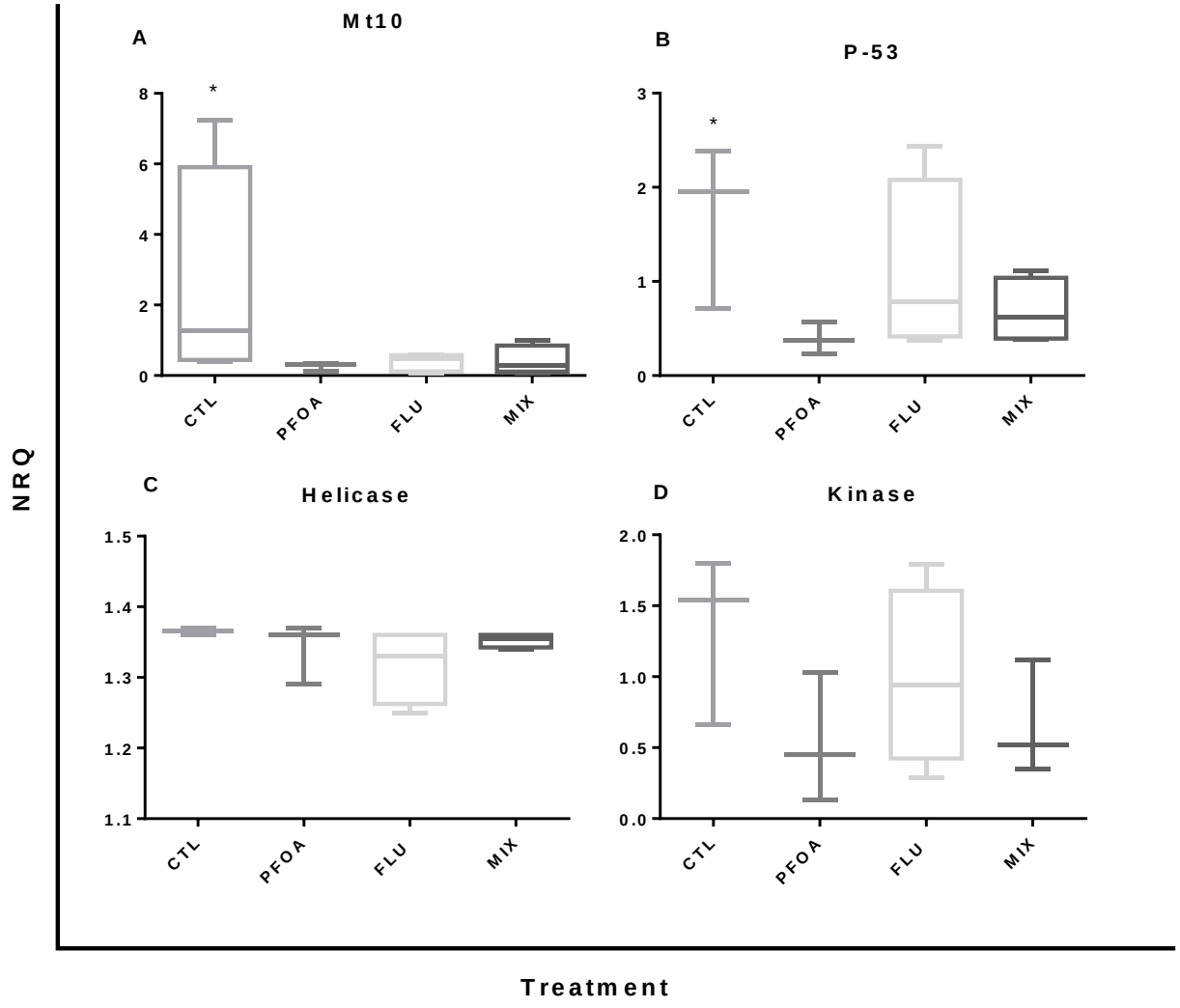

Figure 3.2 Normalized relative quantified (NRQ) gene expression for mussels sampled after 8 days of exposure, where Asterix* indicates a significant difference compared to unexposed samples. Data presented as median, quartiles and 10-90 percentiles. A) expression of *Mt10*. B) expression of *p53*. C) expression of *RNA helicase*. D) expression of Kinase (Krs).

Comparing gene expression within the same treatment groups at different time points showed a significant alteration in the expression of *p53*. Kruskal-Wallis indicated a significant decrease in PFOA-exposed mussels ($p=0.02$), between 4 days of exposure and 16 days of exposure (Dunn, $p=0.03$, $n= 4$).

4. Discussion

In the present study the potential genotoxicity of Perfluorooctanoic acid (PFOA), fluoranthene and the combined effects of the two were examined in haemocytes and gill cells of the bivalve, *Mytilus edulis*.

4.1 DNA damage

DNA damage in haemocytes of *M.edulis* following exposure to the chemicals, and the vehicle control (ethanol exposure only) were analysed by the comet assay with and without enzyme to assess potential DNA breaks and oxidative DNA lesions. The assay has previously proved to be a useful tool for measuring the amount of DNA damage in blue mussel haemocytes, exposed to genotoxic pollutants (Rank et al. 2003; Taban et al. 2004; Haldorsson et al. 2005; Hagger et al. 2005; Jha et al. 2005; Mamaca et al. 2005; Rank et al. 2005).

The results of the comet assay showed a substantial degree of DNA percentage in the comet tail (18-58%) in all samples, including vehicle control and unexposed mussels. Comparing results within a treatment group, between sampling days, revealed significant differences only for the fluoranthene-exposed mussels between 8 and 64 days of exposure. All samples from day 64 must be excluded due to bacterial growth in the tanks.

It is difficult to say if, and how much of the tail DNA intensity in exposed mussels that were caused by background damage and/or ethanol exposure, as unexposed samples had a large level of DNA damage (median tail DNA 41%). Due to the amount of tail DNA in unexposed samples, a comparison of exposed and unexposed samples could not be made. It makes it therefore difficult to interpret the effects of PFOA and fluoranthene in *M.edulis.*

The background level in unexposed mussels masked the analysis of fluoranthene and PFOA to cause DNA damage for *M.edulis*. Other research on the genotoxic effects of these contaminants are varied. Studies have indicated bioaccumulation of PFCs in marine organisms (Liu et al. 2011), and genotoxic effects for organisms exposed to PFOA (Yang 2010; Moussa et al. 2011). However, studies by Hoff et al. (2003) could not find any significant induction of DNA SSBs compared to control in common carp (*Cyprinus carpio*). Genotoxic effects were also not found in the study by Florentin et al. (2011), where exposure of PFOA to human HepG2 cells, after 1 and 24 hour, gave no increase in DNA SBs. In a study by Liu et al.

(2011) they found minor toxic responses in the green mussels, *Perna viridis,* using the comet assay, suggesting that this resulted from low bioaccumulation of PFOA.

There are not many studies investigating the effects of fluoranthene in bivalves. Even though there was no indication of fluoranthene causing DNA damage in this study other studies have shown fluoranthene to cause DNA damage in marine invertebrates (Palmqvist et al. 2003; Morin et al. 2011) and haemocytes of flounder and sole (Woo et al. 2006; Wessel et al. 2006), respectively. The extensively more studied PAH, benzo[a]pyrene (BaP), have shown to produce DNA damage in mussels and oyster cells (Nacci et al. 1996; Steinert et al. 1996; Mitchelmore et al. 1998a). Other studies have revealed that BaP caused DNA adducts (Venier and Canova 1996) and DNA SBs (Bihari et al. 1990) in *M.edulis.*

The results obtained by the comet assay indicated high inter-individual differences in DNA damage for all treatment groups. This variability in the toxic response is attributed to physiological differences that vary among individuals, it includes; enzyme activity responsible for activation and detoxification of exogenous compounds, antioxidant defence, metabolic activity, genetic susceptibility, DNA repair efficiency, and others (Lemaire and Livingstone 1993; Chaney and Sancar 1996; Akcha et al. 2003a; Mitchelmore and Chipman 1998ab). Heterogeneity in DNA damage between individuals can also be related to variation in cell age and cell cycle (Akcha et al. 2004).

4.1.1. Vehicle (ethanol) exposure

Ethanol was used as a carrier solvent and most likely caused an indirect and direct effect on the level of DNA damage for mussels exposed for 64 days, and vehicle control exposed for 4 days. In the present study, fluoranthene-exposed mussels were the only treatment group that showed significant differences in percent tail DNA between sampling days. This was between 8 days and 64 days of exposure. The vehicle control was also significantly different between 16 and 64 days of exposure. However, as mentioned, the level of DNA SBs after 64 days of exposure in all treatment groups, including vehicle control, cannot be attributed to the chemicals. A reduction in dissolved oxygen concentration was likely to be the reason, believed to be caused indirectly by the solvent carrier. Ethanol and other organic solvents can lead to additional carbon sources for microbial growth when used in an exposure system over a longer period (ECETOC 1996). Reduced oxygen levels in organisms can induce oxidative stress, which is known to cause damage to the DNA and other cellular components (Pacifici 1991). Liepelt et al. (1995) supported the consequence of oxidative stress by demonstrating that subtle changes in oxygen level, in ambient water, could have a profound effect on the DNA integrity in gill cells of rainbow trout. Mustafa et al. (2011) also suggested that low oxygen levels could induce DNA SBs in fish. It is therefore

reason to believe that non-toxic exposure, following low oxygen levels could affect the background level in mussels, as well.

In addition to causing an indirect effect, ethanol may also have caused a direct effect by inducing DNA damage in the vehicle control after 4 days of exposure. These mussels had a relatively large inter-individual variance compared to mussels exposed for 8 and 16 days. No studies have investigated the possibility of ethanol causing DNA damage in bivalves, however, studies have shown that ethanol, even at low concentration (<20 µl/l), caused a significantly lower growth and a reduced reproduction in juvenile snails after *in vivo* exposure (Lecomte et al. 2013). It is reason to believe that the increased DNA damage for individual mussels, exposed to ethanol, could have been avoided if the mussels were acclimatized to the solvent, prior to the experiment. This is likely, due to the reduced inter-individual variance seen in mussels after 8 and 16 days of exposure. It is therefore fair to consider that the ethanol-exposed mussels were capable of adapting to the contaminated environment and prevented ongoing genotoxic damage.

4.1.2 DNA damage in unexposed mussels

Knowledge of baseline (untreated cells) DNA damage is crucial for the interpretation of the result. Collins (2004) stated that the best way to know whether cells are in a satisfactory condition for comet assay analysis, is that the baseline should not produce comets with a background level above 10% tail DNA intensity. This could mask the result and the relationship between cell damage and DNA damage caused by exogenous agents, making it difficult to interpret the results. In this study, DNA damage in untreated samples was surprisingly high, with a median tail DNA of 41 %. Causes for the high background DNA damage are unknown.

The high amount of DNA damage in unexposed samples may be attributed to endogenous and/or mechanical damages during sampling and isolation of the cells (Olive et al. 1992; Nacci et al. 1996). The method for sampling and cell preparation was performed the same way for each sampling day. It is therefore questionable if damage to DNA in unexposed samples were due to the protocol and sampling technique, especially since a significantly reduced intensity of tail DNA was seen in vehicle control at sampling day 8 and 16, and a non-significantly reduced intensity of tail DNA was seen on day 4. A study by Mitchelmore et al. (1998b) found that the control cells of *M.edulis* digestive glands, demonstrated a higher level of SBs compared to many vertebrate cell types. Singh et al. (1989) found increased levels of alkali-labile sites in certain cell types with highly condensed chromatin, suggesting that the number of SBs were not due to artificial damage or endogenous SSBs, but rather a feature of DNA packing and

background alkali-labile sites. Increased control levels was also found by Nacci et al. (1992), where a DNA alkaline unwinding assay was used to detect DNA strand breaks in gill tissue of marine mussels. Other studies have also found high control levels for invertebrates, using DNA SB assays, and the protocol was not assigned as a reason for the large amount of DNA damage for control samples (Everaarts 1995; Steinert 1996; Mitchelmore 1997, 1998a).

M. edulis, being an aerobic organism, is constantly exposed to endogenous and exogenous oxygen radicals and other oxidants. Even though anti-radical defence systems are present, these oxygen radicals can induce oxidative stress, potentially causing damage to the DNA and other cellular components (Pacifici 1991). Seasonal variation in the antioxidant defence system could be an attributing factor to increased oxidative stress level during low antioxidant activity. Viarengo et al. (1991) explained this by a change in metabolic status of the mussel, depending on gonad ripping and food accessibility, thereby influencing the effects from oxidative stress. This may explain some of the reason for the background DNA damage in the haemocytes, seen in this work.

The mussels were sexually mature (50-70 mm in length). No correlation between mussel size and DNA strand breaks was investigated in this study, but other studies have shown that mature organisms may have a significantly larger amount of DNA damage compared to juvenile organism (Akcha et al. 2003a; Rank et al. 2005). Akcha et al. (2003a) explained this by adults having a higher potential of biotransformation compared to juveniles. Studies carried out by Giovannelli et al. (2003) on rats and Pruski and Dixon (2003) on the vent mussel, *Bathymodiolus azoricus*, also suggested that age can influence DNA damage, suggesting that DNA repair capacity may be reduced with age/size. This suggests that the age of the animals may have influenced the level of background DNA damage seen in this study. On the other hand, research by Wilson et al. (1998) using *M.edulis* and Hartl et al. (2004) using the clam, *Tapes semidecussatus* found no relationship between size/age and DNA damage in gill cell and haemocytes, respectively.

Spawning was not observed during the experiment, but the unexposed mussels were sampled (unintentionally) just prior to spawning as sperm and egg cells were easy to notice in the haemolymph, examined under a microscope. Hartl et al. (2004) showed that the clam *T.semidecussatus*, exposed to genotoxic compounds and with well developed gonads showed a significant elevation of DNA damage in gill and digestive gland cells, demonstrating a sensitivity towards contaminants. This was explained by impaired DNA repair capacity due to reproductive activity. Research by Sastry and Blake (1971) have suggested that diversion of stored energy to gametogenesis could result in less energy for processes of

somatic growth and baseline metabolic maintenance, such as DNA repair. Consequently, depending on gonad ripping, mussels may be more susceptible to contaminant influences, which may have contributed to the magnitude of DNA SB in unexposed samples, seen in this experiment.

Another possibility, for the high background level, could be due to the seawater received by the unexposed mussels. Very few coastal areas are without anthropogenic impact, and sediments and biota are often contaminated with chemicals to some degree (Rank et al. 2005). It might be that the water received from the fjord, within the sublittoral zone, could have been contaminated with pollutants causing a genotoxic effect in the unexposed mussels. Nonetheless, there is no reason to believe that the seawater at this site was any more contaminated than the original site where the mussels were collected from.

4.2 Oxidative stress

One of the aims of this project was to study the possible induction of oxidative DNA damage in haemocytes, following in vivo exposure to PFOA, fluoranthene and the combination of these two.

In addition to the formation of DNA damage by exogenous exposures to contaminants, oxygen radicals can also modify the DNA. These radicals may ascend as a product of endogenous processes, and alternatively from oxidative stress generated as a by-product of metabolism of xenobiotics (Farmer et al. 2003). A well known type of oxygen radical is reactive oxygen species (ROS), which may result from endogenous metabolism, or it may be produced during redox cycling or other free radical interactions related to organic pollutants and metabolites (Mitchelmore and Chipman 1998b). The modified Comet assay protocol, which targets oxidized DNA bases by treating the cells with a specific enzyme, have previously been shown to enhance the sensitivity and specificity of the method (Dusinska and Collins 1996). The use of Formamidopyrimidine DNA glycosylase (FPG) in conjunction with the assay facilitates the detection of oxidative DNA lesions, and can give insights concerning the comparative contribution of oxidative DNA damage to overall DNA damage (Gielazyn et al. 2003).

A significant increase in tail DNA intensity was seen between cells incubated with enzyme and cells only incubated with enzyme buffer for unexposed mussels, indicating that the enzyme did induce FPG sensitive sites. This conform with previous findings that FPG detects altered purines especially

8-Oxoguanine (Collins 1996; Collins et al. 1998), and also identifies apurinic (AP) sites and open ringed N-7 guanine adducts (Akcha et al. 2003a; Li et al. 1997; Tchou et al. 1994).

The high background damage made it difficult to interpret the results from the FPG treatment. This probably gave a substantial amount of oxidative DNA damage in unexposed samples. Comparing the exposed groups to the unexposed group revealed a significant reduction in oxidative damage for PFOA-exposed mussel and fluoranthene-exposed mussels after 64 days of exposure. As explained in section 4.1.1, all samples from day 64 must be ignored due to bacterial growth in the tanks. The vehicle control was also compared to the unexposed group, which showed a significant decrease in FPG sensitive sites after 4 days. When evaluating oxidative stress due to exposure duration there was a significant difference for mussels exposed to PFOA. Mussels exposed for 16 days had significantly increased oxidative DNA damage compared to mussels exposed for 4, 8 and 64 days.

As explained, the amount of background damage in unexposed samples made it difficult to interpret the results, and masked the result of the chemicals to cause oxidative stress. When comparing the exposed group to corresponding vehicle control, there were only seen an indication of oxidative stress in mussels exposed to PFOA after 16 days.

Based on the result that PFOA was the only treatment that indicated oxidative stress and the lack of evidence for direct genotoxicity of PFOA (Anderson et al. 2008), it is reasonable to assume that the induction of excess ROS production could be the main pathway of PFOA to induce DNA damage. Oxidative stress were also seen in the green mussel, *perna viridis,* exposed to PFOA in the study by (Liu et al. 2013) and proposed that an excess production of ROS could be the main toxic pathway. Other studies have found that PFOA can generate ROS and induce oxidative DNA damage in human HepG2 cells (Lui et al. 2007; Hu et al. 2009). Eriksen et al. (2010) and Yao and Zhong (2005) measured FPG-sensitive sites in the DNA of human liver, using the comet assay, with contradictory results. Eriksen et al. (2010) found that PFOA gave an increase in ROS production, but there was no indication of DNA damage such as SBs, alkali-labile sites or FPG sensitive sites (oxidative DNA damage). Zao and Zhong (2005), however, concluded that PFOA gave oxidative DNA damage in HepG2 cells, as a significant increase in ROS and 8-hydroxy-2'-deoxyguanosine (8OHdg) levels were observed in cells exposed to PFOA. Another study by Lui et al. (2007) indicated that PFOA increased the production of antioxidants such as Superoxide dismutase (SOD), Chloramphenicol acetyltransferase (CAT) and Glutanion reductase, while decreasing Glutanion peroxidase and Glutanion S-transferase (GST) in hepatocytes of freshwater fish, due to the production of

ROS in PFOA exposed cells. An Increase in 8-hydroxyde guanosine in liver of rats have also been detected, suggesting that PFOA induced the level of ROS production (Takagi et al. 1991). Conversely, in the study by Florentin et al. (2011) there was no indication of PFOA causing ROS production in human HepG2 cells, at the concentration tested.

Studies have shown (Coles et al. 1994; Cavalieri and Roger 1995) that PAHs, such as fluoranthene, not only form adducts by binding to endogenous molecules, but can also produce oxygen radicals. As opposed to PFOA, fluoranthene can be metabolized by aquatic organisms and are therefore capable of generating ROS (Stegeman 1985; Winston et al. 1988), which can lead to oxidative stress in marine animals (Hannam et al. 2010; Wessel et al 2010) that may exert oxidative damage to proteins, lipids and DNA (Apel and Hirt 2004). However, this study gave no indication of fluoranthene causing oxidative DNA damage in haemocytes of *M.edulis.*

4.3 Gene expression

To evaluate whether the test compounds could exert any effects at the transcriptional level, the gene expression of *Mt10, p53, RNA helicase* and *stress responsive kinase (Krs)* was evaluated for mussels exposed for 0, 4, 8 and 16 days. Altered gene expression for mussels have previously been used for the assessment of cellular responses to genotoxic pollutants (Lemoine et al. 2000; Brown et al 2006; Dondero et al. 2006ab)

P53 was significantly altered when singly exposed to PFOA and fluoranthene. Comparing the gene expression after 4 days of exposure to the gene expression at time 0, showed PFOA-exposed samples to have significantly increased expression of *p53*, as did fluoranthene-exposed mussels. The gene expression of samples exposed to PFOA for 8 days compared to the gene expression of the corresponding vehicle control also indicated a significant increase of *p53*.

p53 has a function in tumour suppression and regulates various cellular events, such as cell cycle, DNA repair in response to DNA damage, protective mechanism or if necessary the cessation of cell division and induction of apoptosis. It therefore plays an important role in preserving genomic stability (Hoeijmakers 2001; Dlamini et al. 2004). Under abnormal events during the cell cycle, for instance genotoxic stress, the expression of *p53* is up-regulated in order to arrest the cell cycle or to induce apoptosis in the altered cell (Mihara et al. 2003; Harms et al. 2004). It is recognized that PFOA may cause

increased levels of ROS in mammalian cells (Panaretakis et al. 2001; Hu et al. 2009) and invertebrates (Liu et al. 2007). ROS are produced continually in living cells, and are essential in maintaining cell function. However, an imbalance between formation and neutralization of such reactive species can induce oxidative damage (Valavanidis et al. 2006). This can lead to both acute toxic and genotoxic effects if these antioxidant defences are overcome (Palmqvist et al. 2003; Palmqvist et al. 2006), causing a series of complex biological responses, including damage to the DNA (Binelli et al. 2009; Al-Subiai et al. 2011). Rämet et al. (1995) found a temporal link between DNA adducts and an increased *p53* protein level suggesting a direct cause-effect correlation between *p53* and adducts. Another study found an up-regulation of *p53* in embryo of zebrafish exposed to PFOS, a related compound to PFOA (Shi et al. 2013). The identification of *p53* genes in soft tissues of *Mytilus spp*. have been reported (Ciocan and Rotchell 2005; Muttray et al. 2005), but the role of this protein family in marine invertebrates is not clear. Data from studies on haemocytes of *Mytilus arenaria,* suggest a similar role as in mammals (Kelley et al. 2001). It is therefore likely that the up-regulation of *p53* seen in the present study is a response for potential DNA damage from the induction of ROS and other oxygen radicals.

Certain metabolites produced during biotransformation can cause the toxicity associated with PAHs, such as genotoxicity (Akcha et al. 2003b; Wessel et al. 2012). In mollusks, the metabolism of PAHs occur mainly through radical oxidation involving ROS (Stegeman 1985; Winston et al. 1988). As fluoranthene may be metabolised by aquatic organism and generate ROS, which can lead to genotoxic effects, it is reason to believe that not only PFOA, but also fluoranthene may have induced the expression of *p53,* through the generation of oxidative radicals, seen after 8 days of exposure. Studies have shown that *p53* can be up-regulated in cells exposed to PAHs, like BaP, in mammals and fish (Binkova´ et al. 2000; Brzuzan et al. 2006).

The gene expression for samples exposed for 4 days compared to the gene expression at time 0, displayed a significantly increase in the regulation of *Krs* for PFOA-exposed mussels. *Krs* exhibited steady expression rates after 8 and 16 days of exposure, indicating an adaption to the PFOA treatment.
It has been verified that cells respond to stress, such as chemical treatment, by activating protein kinase (Davis 1994). Stress responsive kinases play a crucial role in the regulation of cellular signalling pathways in response to external and internal stress. A study by Pombo et al. (1996) reported that the activation of human *stress responsive kinase* was coupled to oxidative stress. Dondero et al. (2006a) concluded that

the homologue gene of *Krs* was up-regulated in mussels sampled from a polluted site. This was explained as a response to increasing oxidative stress in individual samples.

The expression of *Mt10*, known to be affected by oxygen stress (Bauman et al. 1991), showed no significantly alteration when compared to the gene expression at time 0. Comparing the gene expression for vehicle control to the gene expression at time 0 showed a significant alteration of *Mt10*. The expression of *Mt10* was significantly elevated in vehicle control after 4 days, while the expression of *p53* was significantly elevated after 8 days of exposure. *Mt10* and other metallothioneins (Mt) are used extensively as molecular biomarkers for toxic metals, but also for oxidative stress (Valavanidis et al. 2006). Although it is known that hydrogen peroxide (H_2O_2) and other oxygen radicals can stimulate Mt mRNA synthesis (Dalton et al. 1994), Dondero et al. (2005) found no effect on the gene expression of *Mt10* for mussels (*Mytilus galloprovincialis*) exposed to Hydroxyl radicals.

As both *Mt10* and *p53* showed a significantly increase in their regulation for vehicle control compared to unexposed mussels, it is reason to believe that ethanol may have caused this effect. Other studies have reported an induced synthesis of Mt in hepatic cells for mice given ethanol orally (Waalkes et al. 1984; Bracken and Klaasen 1987). The up-regulation was seen after 4 days of exposure, while 8 and 16 days of exposure showed a steady expression rate, indicating an adaptive response to ethanol.

RNA-helicase is in eukaryotic cells involved in modification of RNA secondary structures, ribosome assembly and translation initiation (Linder et al. 2003). It has previously been shown that mussels challenged with oil exposure have reduced expression of *RNA helicase* (Dondero et al 2006b). However, no significantly alteration was indicated in the expression of *RNA helicase* in the previous study.

4.4 Co-exposure of PFOA and fluoranthene

Most genotoxic studies to date have focused on single-substance exposures. No studies have investigated the combined genotoxic effects of PFCs and PAHs in marine organisms. There are likewise not many studies investigating the effects of PFOA or fluoranthene in marine invertebrates. One knows little of the synergetic effects of PFOA, though, PFOS, a PFC similar to PFOA, have shown to increase the genotoxicity of other chemicals in mammals. (Jernbro et al. 2007). There is, however, no indication in this study that the binary effect of PFOA and fluoranthene could lead to increased genotoxicity, in the

form of DNA strand breaks or oxidative lesions. The same conclusion can be drawn from the RT-qPCR analysis, where the expression of genes, related to oxidative stress, and general stress, in gill cells, did not differ significantly compared to unexposed or vehicle control.

5. Conclusions

There was a substantial degree of DNA damage in all treatment groups, including vehicle control and unexposed samples. The amount of DNA damage in unexposed mussels masked the effect of the exposure study. The protocol was not assumed the responsible cause, as there was a smaller amount of DNA damage in vehicle control.

Comparing exposed mussels to vehicle control gave no indication of significantly elevated DNA damage. Ethanol probably caused bacterial growth in the tanks after 64 days of exposure and therefore all of the samples from this day was ignored.

A significant elevation of oxidative DNA damage in Perfluorooctanoic acid (PFOA) exposed samples after 16 days of exposure was evident, compared to vehicle control, and 4 and 8 days of PFOA exposure.

Altered gene expression was used to evaluate the compounds effect on the transcriptional level in the gills. A significant up-regulation of *p53* mRNA level after 4 and 8 days of PFOA exposure, and after 8 days of fluoranthene exposure indicated genotoxic stress, as both PFOA and fluoranthene are known to cause ROS and other oxygen radicals. There was no significant alteration in gene expression of *p53* after 16 days, suggesting an adaptive response and prevention of ongoing oxidative damage.

The relative expression of *stress responsive kinase (Krs)* had significantly increased after 4 days of exposure to PFOA. There is a possibility that this was a reaction to oxidative stress as previous research have couple the up-regulation of *Krs* to oxidative stress, and, as mentioned, PFOA can produce oxidative radicals, causing oxidative stress. No significant alteration in the expression of *Krs* was seen after 8 and 16 days, indicating an adaption to the PFOA exposure.

Even though the increased expression of *p53* and *Krs* could suggest a response to oxidative stress, the gene *Mt10*, known to be affected by oxidative stress, showed no significant alteration in its level of expression. The same was observed for *RNA helicase*.

The results from the comet assay and RT-qPCR gave no indication of increased genotoxicity for mussels co-exposed to PFOA and fluoranthene.

It is reason to believe that ethanol exposure caused an effect in both haemocytes and gill cells. However, the response appeared to be adaptive as the vehicle control after 4 days of exposure had an increased inter-individual variance in DNA damage compared to 8 and 16 days of exposure. The expression analysis

revealed that mussels exposed to ethanol had a significantly increased level of *Mt10* and *p53* after 4 days and 8 days of exposure, respectively, but not after 16 days.

6. Further work

Few experimental studies have investigated the interaction between PAHs and PFCs in marine environments, and more studies in this field are needed to increase the insufficient knowledge.

There was a substantial amount of DNA damage for unexposed (control) mussels. The data from the comet assay needs therefore further validation using new control samples for the investigation of DNA damage in haemocytes.

A follow-up of this study would be to consider if there is any seasonal variation in the response to Perfluorooctanoic acid (PFOA) and fluoranthene. The mussels in the experiment had well developed gonads, possibly resulting in an increase in DNA damage and oxidative stress. Taking samples at another time of the year could have revealed if this was the case. In addition, it would have been interesting to investigate sex-dependent differences in DNA damage and oxidative stress.

As the result suggested, oxidative stress could be an important toxic pathway of PFOA and fluoranthene. However, in the present study only a few genes related to oxidative stress were tested. Other oxidative stress-related genes (e.g. Catalase, GST-pi, GSH-peroxidase, HSP27/70, SOD) could be included in other studies to gain deeper insight of PFOA- and fluoranthene-induced oxidative toxicity.

It would be of interest to test the genotoxic effects of ethanol for mussels and other marine invertebrates since the in vivo exposure-results indicate an effect in both haemocytes and gill cells. This could help optimize the concentration of ethanol when used as a carrier solvent, ensuring further work from not being influenced by ethanol exposure.

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Appendix

Appendix A: Solutions and media

Comet assay

Collins` buffer (Enzyme reaction buffer)

Chemicals	Quantity (5L)	Final concentration
Hepes (Mw: 238.31 g/mol)	47.65 g	40 mM
KCL (Mw: 74.56 g/mol)	37.25 g	0.1 M
Na_2EDTA (327.24 g/mol)	0.93 g	0.5 mM
dH_2O	pH adjusted to 7.6, dH_2O added until 5 L	

Phosphate Buffer Saline (PBS)

Chemical	Quantity
NaCl	8.5 g
Na_2HPO_4	0.85 g
KH_2PO_4	0.54 g
dH_2O	pH adjusted to 7.4 and dH_2O added until 100 ml

Lysis Buffer- stock solution

Chemicals	Quantity (2670 ml)	Final concentration
dH_2O	2100 mL	--
NaCl (58.44 g/mol)	438.30 g	2.8 M
NaOH (40.0g/mol)	24.00 g	0.244 M
$Na_2EDTA * 2H_20$ (372.2 g/mol)	111.66	0.112 M
Trizma base (121.2 g/mol)	3.66	0.0113 M

pH adjusted to 9

Lysis buffer- working solution

Chemicals	Quantity (600 ml)
Lysis stock solution	534 ml
dH_2O	60 ml
Triton X-100	6 ml

Electrophoresis Buffer – stock solution

Chemicals	Quantity (5L)	Final concentration
NaOH (Mw: 40.0)	600 g	3M
Na_2EDTA (Mw: 372.24	18.61 g	10 mM
dH_2O	Adjust volume when dissolved	

Electrophoresis Buffer – working solution

Stock	dH2O	Concentration
200 ml	1800 ml	0.3 M (NaOH) 1.0 M (Na_2EDTA)

pH adjusted to 13.2 by adding 6 mL HCL per liter (4 C˚)

Neutralizing Buffer

Chemicals	Quantity (2L)	Concentration
Trizma Base (121.2 g/mol)	96.96 g	0.4 M
dH_2O	Adjust pH to 7.5 and volume to 2 L	

75% LMP Agarose (pH 7.5)

Chemicals	Quantity
LMP	75 mg
PBS + 10 mM EDTA	10 ml

75 mg LMP dissolved in 10 ml PBS and 10 mM EDTA, heated to boiling point until dissolving of agarose and kept at 37 C˚ on a heat block.

Staining solution (SYBR® Gold)

Chemicals	For one GelBond film	Concentration
TE-Buffer	25 ml	--
SYBR Gold (Pre-diluted aliquot)	20 ul	Approximately 1:10000

Tris EDTA (TE)-Buffer

Chemical	Quantity (1 L)	Final concentration
0.5 M Tris-HCl (pH 8)	20 ml	10 mM
0.5 M EDTA (pH 8)	2 ml	1 mM
dH_2O	Adjust to pH 8, and volume to 1 L	

Appendix B: The formula of relative quantification in RT-qPCR

A) Relative quantification of the threshold value of the target gene and reference gene

- *RQ = E^(min Ct-Ct(current sample))*

RQ: relative quantification E: primer efficiency, Ct (current sample): Ct value of present sample, min Ct: minimum Ct value for all samples within present primer.

B) RQ of target gene normalized against RQ of reference gene

- *NRQ = RQ (target gene)/RQ (reference gene)*

NRQ: Normalized relative quantification.

C) The median of the NRQ value of each sample is normalized to the control samples.

Fold change = median NRQ(sample)/median NRQ(control)

This gives the fold-change values of each sample

Appendix C: Lab equipment and chemicals

Products and chemicals

Product	Supplier	Country
Agilent RNA 6000 Nano Kit	Agilent Technologies	USA
Absolut alcohol (100% ethanol)	Arkus	Norway
Bovin serum albumin (BSA)	Sigma Aldrich	USA
Dimethyl sulphoxide (DMSO)	Merck	Germany
Calcium chloride (CaCl)	--	--
Distilled water	Locally produced	Norway
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich	USA
Formamidopyrimidine-DNA-glycosylase (FPG)	Locally produced (NIPH)	Norway
Gelbond® film	Cambrex	USA
Hepes	Sigma Aldrich	USA
Hydrogen Chloride (HCl)	Merck	Germany
Isopropanol	--	--
LMP Agarose	Sigma Aldrich	USA
mirVana miRNA isolation kit (Ambion®)	Life technologies	USA
Na_2EDTA	Sigma Aldrich	USA
Na_2HPO_4	Merck	Germany
Natrium hydroxide (NaOH)	Merck	Germany
Phosphate buffer solution (PBS)	Locally produced	Norway
Potassium chloride (KCl)	Merck	Germany
Potassium dihydrogenphosphate (KH2PO4)	Merck	Germany
Potassium hydroxide (KOH)	Merck	Germany
RNA nano kit	Agilent Technologies	USA
Sodium Chloride (NaCl)	Merck	Germany
Sodium hydrogenphosphate (Na2HPO4)	Merck	Germany
Sodium hydroxide (NaOH)	Merck	Germany
SYBR® Gold	Invitrogen	USA
Transcriptor first strand cDNA synthesis kit	Roche applied science	Germany
Trizma® HCl	Sigma Aldrich	USA
Triton-X	Sigma Aldrich	USA
Trizma® base	Sigma Aldrich	USA
Transcriptor first strand cDNA synthesis kit	Roche applied science	Germany

Lab equipment and manufacturers

Products	Manufacture	Country
96-well PCR plate	Thermo Scientific	Germany
Agilent 2100 Bioanalyzer	Agilent Technologies	USA
A312f camera	Basler Vision Technologies	Germany
Analytical scale, AG204	Mettler Toledo	Switzerland
Analytical scale, PB210 S	Sartorius	Germany
Agilent 2100 Bioanalyzer	Agilent Technologies	USA
Comet Assay IV (software)	Perceptive Instruments	UK
Centrifuge 5424	Eppendorf	Germany
Electrophoresis system	Norwegian Institute of Public Health	--
LightCycler® 480 Multiwell Plate 96, white	Roche applied science	Germany
LightCycler® 480 Multiwell Plate 384, white	Roche applied science	Germany
LightCycler® 480 Multiwell Sealing Foil	Roche applied science	Germany
LightCycler® 480 Real-Time PCR system	Roche applied science	Germany
Mastercycler ep Gradient S	Eppendorf	Germany
BX51 microscope	Olympus	USA
Gen5	BioTek	USA
LAB pH meter, PHM 92	Radiometer	Danmark
Mercury Burner	Osram	Germany
Precellys® 24-Dual tissue homogenizer	Bertin Technologies	France
Precellys tubes	Bertin Technologies	France
RNA nano chips	Agilent Technologies	Germany
SynergyMX multimode reader	BioTek	USA
Master cycler Ep S (thermal cycler)	Eppendorf	Germany
Vortex mixer (SA8)	Stuart equipment	UK